

Is it “*Oui* or *Nous ne savons pas*”?

The uncertainty caused by the French referendum on Europe last Sunday could threaten many institutions of European collaboration. Responsibility for avoiding that outcome rests with the British.

FOR France, and for the time being, the outcome of last Sunday's referendum on the Maastricht Treaty is clear enough. France says “YES”. Sadly, the message for much of the rest of Europe is “DON'T KNOW”. After last week's upheavals, which forced Britain and Italy out of the European Monetary System, it would not have made much difference if the French majority in favour of Maastricht had been less marginal. The goal behind Maastricht, negotiated just a year ago, has been thoroughly obscured. Those effectively expelled last week from the dream of European union, like Denmark (which opted out in June), will not be able to rejoin quickly. The British government's intention to fight for ratification of Maastricht in the House of Commons now looks less likely to succeed, given the growing dissent among its own supporters. But the surviving members of the club will have learned from last week's events that the foreign exchange markets will be watching their currencies like hawks, between now and the introduction of a single currency, for signs of divergence from the fiscal and monetary targets set by Maastricht. They have every reason to hurry up. Can this gulf between the two groups be bridged?

Even those who hope that something may be possible should appreciate that the Maastricht Treaty as it stands falls far short of the ideal. The intended European Union was meant to act in unison on most of the matters that concern governments, but (as specified) it lacks a means by which its managers can make policy by democratic means. The elected European Parliament would remain a toothless animal; the decisions that matter would continue to be made in the fashion that has given Brussels a bad name, by groups of national ministers with sectional and often conflicting interests meeting behind closed doors and working against the clock. The Maastricht Treaty itself is the outcome of such a process of systematic compromise. No wonder that nearly 49 per cent of those who voted on Sunday found it unconvincing.

The centrepiece of the treaty, the proposed European currency backed by an independent bank on the model of the Bundesbank, is similarly flawed. The notion a year ago was that, because the German central bank has delivered sound money for 30 years, an independent central bank would be able to do the same for the whole of Europe. The snag is that the pursuit of sound money can be painful for those whose economies are weak, as last week's events have shown. Is that what Europe wants, especially in a transition period such as that which lies ahead?

The misfortune is that all this could and should have been foreseen. The pity is that the present uncertainty over Europe's future is bound to be a cruel disappointment of many hopes for closer European collaboration. One of the heartening developments of the past few years has been that the European scientific enterprise has been made much more cohesive and effective by researchers' willingness to work at each other's laboratories, language difficulties notwithstanding. It is true that none of this need stop because of last week's debacle on the foreign exchanges. The Single Market, after all, will remain in being. But it is unthinkable that Europe's outsiders will willingly continue to contribute to the strengthening of the common institutions that have begun to oil the wheels of productive collaboration. The European Commission's research programme, with all its faults, looks a likely casualty.

Can the gap be bridged? The responsibility for finding a solution rests with the British who hold the presidency of the EC until next year. Tactically it would make sense if the British energetically set out to make an accommodation between Denmark and the EC's YES members in the hope that the result will also make Maastricht more acceptable to the House of Commons. But that strategy may not work. The British government looks a little shell-shocked after last week's events, and may not have the spirit. Worse, France, Germany and the Benelux countries may be too impatient — and too fearful of the currency markets — to hang about. □

Saving on health

Two prosperous countries in the West — Germany and the United States — are in trouble over health costs.

DOES more health care mean better health? That is the question raised the other day by Mr Horst Seehofer, the minister of health in the German federal government. His immediate difficulty is a nearly twofold increase of Bonn's subsidy of the German health service between 1991 and 1992, in part a delayed consequence of unification. So the government plans that pharmaceutical prices shall be reduced (by law) and that health insurance costs should be increased. In the United States, on the other hand, the cost of health care has become an election issue. President George Bush insists that the market will arrange that what people pay for health care is what they want to, but Mr Bill Clinton, his



Democrat challenger, wants to "cap" some costs and was able to other day to claim the support of the American College of Physicians (not to be mistaken for the American Medical Association), which has advocated agreements to contain some medical costs — the fees charged by physicians, for example.

Developments such as these are at present unavoidable. In what was then West Germany, people in 1990 spent no less than 10 per cent of their gross national product on health care, amounting to some DM4,770 per head of population. Alarming, the rate of increase of spending has been running at just under 10 per cent a year — faster than the rate of economic growth in Germany's best years some time ago. The burden carried by Germany's wage-earners is substantial; health insurance alone accounts for some 13 per cent of average income. In the United States, more of the burden is shouldered by companies by way of employment-related insurance plans, and so is less conspicuous to voters, while costs are increased to an unquantifiable degree by the cost of malpractice suits (and that of the insurance physicians must buy). What will happen to these bills when the armoury of new diagnostic tests on which every other biotechnology company is working eventually reaches the market?

The difficulty is that caps and across-the-board cuts in prices are bound to be arbitrary and even inequitable. Physicians with exceptional skill or specialism will be insufficiently regarded (and others will be discouraged from following in their footsteps). And arbitrary ceilings on drug prices will penalize innovative manufacturers more than others. Are there not simpler ways of containing the costs of health care?

In both Germany and the United States, the 'market' in health care is inefficient, to say the best of it. People who are sick naturally seek a cure quickly, and do not hunt for the best bargain in the field even if they have an incentive to do so (which is often not the case). Equally, the physicians to whom they turn do not expect to have to haggle over prices in advance.

But why not require that physicians should publish to their patients and other interested parties the prices they will charge for the various services they are qualified to provide? If it were then also required that patients should pay a proportion of the cost, there would at least be a chance to see whether the market of which Bush speaks would function. And there might then be the funds left over to pay for health care for those who cannot now afford it. □

Rebuild research now

The temptation to cut back on research in the current financial crisis should be resisted.

PITY poor Britain. Two years after the pound sterling was put into the European Exchange Rate Mechanism, six years after its Chancellor of the Exchequer started tracking the Deutschmark on the foreign exchanges and a dozen years after embarking on a serious effort to put its money affairs

in order by cutting public spending, sterling was last week forced out again by the sheer cost of supporting its value. Italy, in similar case, also of course commands sympathy, even if its new prime minister, Mr Giuliano Amato, has hardly had a chance seriously to tackle his government's public finances. In each case, but especially in Britain, there is a danger that the consequences will include further attempts to save by spending less on science. But the British experience of the past twelve years must surely argue in the opposite direction.

As a percentage of gross domestic product, British government spending on academic and related research is now less than that of comparable European governments. Worse, as the government's own Advisory Council on Science and Technology reported in July (see *Nature* 358, 359; 1992), the shortage of funds has created other problems; salaries in research compare poorly with those in the commercial field, such research spending as there is too thinly spread and, even when universities have been able to make good the shortfall by means of contracts with industry, they are often unable to recover the full costs. All this is well known, of course, although it is valuable that one of the government's own committees now agrees. But how, the moneybags will ask, can more spending on research help a country such as Britain out of the tight corner in which it finds itself?

Although the gestation period of important and marketable industrial innovations may be long and unpredictable, the research system can yield the products which are its primary purpose — young people educated in research — quickly and reliably. In the British system, a new graduate can still earn a research degree in three years or so, becoming in the process a potentially more creative member of a modern community. Whatever happens now to Europe (and Britain's place therein), this must surely be the worst possible time further to intensify the discouragement of young people from careers in research. Those tempted to act in such a way should reflect that there is at present no way of telling the degree to which an insufficient supply of such people may have contributed to the recent precipitate decline of British competitiveness — the underlying cause of last week's debacle on the foreign exchanges.

With unemployment pushing steadily towards 3 million, and with pressure on the government mounting to devise some quick stimulation of the economy, perhaps by construction projects, a plea that this is not the time to provide further discouragement of research may be mistaken for special pleading.

But that is not the case. On the contrary, the immediate benefits of public spending on research should be little different from the same amount of spending on, say, construction. The difference is that the longer-term benefits will be greater. And although Britain's need to rescue its research may be more urgent than in, say, Italy and Spain (Europe's other beleaguered currency), the same truth applies there: the discouragement of research is pointless in the short run and damaging on a longer timescale. □

French court upholds use of animals, fines activists for theft of baboons

Paris. The French Supreme Court of Appeals last week reaffirmed the right to conduct research on animals and sent an unequivocal message to French animal rights groups that they must operate within the law. In upholding convictions of theft against seven activists who had broken into a CNRS laboratory in 1985 and stolen 17 baboons, the court ends a period of uncertainty over the legal status of such acts in France. Its action is also expected to speed up future prosecutions, including the forthcoming trial of activists accused of stealing more than a hundred animals from an INSERM laboratory in Lyons in 1989 (see *Nature* 339, 407; 1989).

The baboon raid at the CNRS Laboratory of Neurophysiology in Gif-sur-Yvette near Paris in 1985 was carefully planned as a media event. A professional cameraman was invited to film the balaclava-wearing commandos as they 'liberated' the baboons, many of which carried surgically implanted cranial electrodes. Robert Naquet, then director of the laboratory, first heard of the raid in a telephone enquiry from the news agency AFP; by the following evening the images had been broadcast on the national television news.

The seven individuals carried their costly defence through two appeals with outside financial help and the support of such animal-rights celebrities as Brigitte Bardot and television personality Alain Bougrain-Dubourg. They claimed that the action was justified by the poor care being given to animals sacrificed in the pursuit of useless research, and they sought to have the taking of laboratory animals recognized under a new legal category distinct from theft. They maintained that laboratory animals were not material objects, that wild animals could not be possessed and that the CNRS had not demanded their return. (The CNRS says that the case had received enough publicity and that it did not want to inflame public opinion by trying to reclaim the animals.)

Naquet, a frequent target of the defendants' lawyers, refuted accusations that he had practised vivisection, obtained the animals illegally and treated them badly. He told the court that the experiments were intended to increase understanding of epilepsy, that the species of baboon used (*Papio papio*) was naturally epileptic and that no other model was available. The fixing of cranial electrodes, he explained, was a painless procedure carried out under anaesthetic.

The court reaffirmed that animal research is a legitimate activity regulated by law: "The CNRS is a public research organization at the centre of scientific and medical

discoveries that benefit man", it ruled. The court rejected arguments that the end justifies the means and reminded the seven defendants that other groups had accomplished their aims without violating the law (several of the accused were identified by witnesses as members of the Animal Liberation Front, but all strenuously denied this). The court



also refused to consider a new category for the taking of laboratory animals, ruling that the baboons were the property of the CNRS and were the target of a common theft.

The seven activists were given suspended prison sentences of up to six months; some were also involved in the Lyons raid, and will go to prison if convicted of that crime. The court also awarded the CNRS damages of FF252,950 (US\$50,000), legal costs of FF7,000 and a symbolic FF1 for the damage to its image. The court also fined the activ-

ists FF90,000 for delaying Naquet's research by six months. The CNRS is expected to use the precedent to extract additional payments from insurance companies for delays to research caused by fires or similar events.

The victory vindicates the new hard-line approach taken by French researchers against break-ins by animal-rights activists. In the past, research organizations have kept silent out of fear that any response would elicit further negative coverage by the media. The CNRS's legal offensive has been paralleled by a campaign by the Ministry of Research and Space to win over the public to the need for animal research.

In January this year, Hubert Curien, Minister for Research and Space, launched his "10 commandments for animal research" at a press conference. Animal experiments in France are already regulated under a 1987 act based on European legislation, but Curien felt that public concerns needed to be addressed further. To this end, he introduced a uniform animal research policy throughout the various organizations that would include the regular publication of statistics, training for researchers, renovation of facilities, closer contact with the veterinary profession, alternatives to animal experimentation and the requirement that all laboratory animals be obtained from specialized breeding institutes by the end of 1993.

Curien's crusade has weakened the animal activists' monopoly on the media. His "10 commandments" were widely and favourably reported in the French press, as were statistics released last month showing a drop of 24 per cent in the number of animals used in public and private laboratories between 1984 and 1990. **Declan Butler**

Blow struck against US pork-barrel

Washington. The victory may be fleeting, but US Representative George Brown (Democrat, California) last week struck a surprising blow against the time-honoured practice of feathering the research nests of members' home districts.

At issue was the spending of \$94 million on ten academic facilities that had neither been requested by the relevant federal agency nor received peer review but had, instead, appeared at the eleventh hour in an appropriations bill for the forthcoming 1993 fiscal year that includes the Department of Energy. (The bill, among other items, includes \$517 million for the Superconducting Super Collider, some \$133 million less

than the Bush administration had requested.)

Brown, chairman of the House science committee, took advantage of a little-used parliamentary manoeuvre to win a 250:104 vote that stripped out the so-called pork-barrel projects and, in their place, permitted \$100 million to be spent on facilities that have been authorized by Congress and reviewed competitively. Supporters of the projects are expected to find an equally obscure way to try to reclaim the money in the Senate version of the bill, but Brown has made it clear that he may oppose any money bill that contains similarly unauthorized research facilities.

Jeffrey Mervis

Confusion about form and function clouds launch of EC's Decade of the Brain

Munich. Hopes for a cohesive European programme for neuroscience research hang in the balance this week as the European Commission (EC) launches its 'Decade of the Brain'. The early launch — before the programme has been approved by EC member states and before any financial commitments exist — has taken many by surprise, and its unveiling at a specialized meeting on depression has added to the confusion about its general aims.

The initiative follows several failed attempts by the 2,500-member European Neuroscience Association (ENA) to persuade the EC to match the Human Frontier Science Program, begun by the Japanese in 1989 and now international, and the US Decade of the Brain, launched in the same year. Last year, however, EC research commissioner Filippo Pandolfi took up the cause and in February 1992 he appointed a task force of experts from member states to analyse the needs of European neuroscience.

By early summer, the task force had developed a detailed four-part proposal, costing ECU100 million (US\$140 million) a year. Each part of the proposal conforms to the normal structure for EC research funding. A 'networking' section proposes the establishment and funding of projects carried out in several laboratories in different countries. Expansion of existing high-cost research facilities such as PET scanners and primate houses is suggested to allow sharing of services between member countries. A training section provides for postdoctoral fellowships, summer courses and the funding of pan-European meetings.

Some 70 per cent of the funds would be destined for joint collaborations between universities and industry, particularly the pharmaceutical industry which has been criticized for investing less in research than do US drug companies. Costs would be shared equally by the EC and industry.

The fate of the proposal rests with the EC's next five-year plan, known as the fourth Framework, which begins in 1994. Although the Framework's structure is still under discussion, the European parliament, which acts independently of the commission, passed a resolution in July recommending that the commission establish "a specific research programme in neurosciences within the next framework programme".

There is concern, however, that the EC's enthusiasm for the idea may have cooled. Michel André of the EC's research division says that neuroscience is "very unlikely" to be accepted as a core theme of the next five-year funding plan. "We can only propose realistic things", he says. "There are lots of

topics in Framework, not just neuroscience — although the commission does think it is a very important subject."

An alternative to a separate programme is an 'umbrella group' that would oversee neuroscience-orientated projects funded within such broader themes as biomedical research and the human mobility programme to promote networking. The group would eliminate redundancy among projects and merge some programmes. André says that this would allow European research to reach a critical mass that has been lacking despite the estimated 12,000 neuroscientists throughout Europe. Under such a system, neuroscience projects would continue to compete for funds against other research disciplines within each general theme.

Members of the task force are unhappy with the idea of an umbrella group rather than a separate theme, arguing that neuroscience warrants a distinct budget and that the Decade of the Brain would be weak-

ened without it. Scientists at last week's meeting of the European Neuroscience Association (ENA) seemed to support the idea of a Decade of the Brain but were confused about what it entailed and its appearance at the end of a small clinical neuropharmacology meeting. EC officials say that the decision to launch the initiative was made in April, after the programmes of the larger pan-European meetings, such as the ENA, had been fixed. And a Brussels-based meeting was preferred to make possible a royal endorsement, in this case by the Queen of Belgium.

Final decisions about how the European Decade of the Brain will be managed and financed are expected within the next couple of months after the overall Framework plans have been completed. In the meantime, lobbying and arguments will continue in an attempt to avoid criticism, widespread in the United States, that such an initiative is likely to involve more rhetoric than new money.

Alison Abbott

Crossing the border, crossing the ocean

Munich. The European Neuroscience Association (ENA) must undergo radical change to meet its goal of raising the quality of pan-European research to the level of that in the United States, according to scientists at last week's annual ENA congress in Munich, which was attended by only 1,700.

Although Europe has a strong base in neuroscience, it is confined to individual countries and fails to reach the 'critical mass' needed to carry out research most efficiently. Scientists and policy-makers say this isolation is the reason Europe trails behind the United States, and that ENA's efforts to increase cooperation among Europe's estimated 12,000 neuroscientists do not match the success of the US Society for Neuroscience.

The US society's annual meeting regularly attracts at least 15,000 participants, including several thousand Europeans who prefer to cross the Atlantic than to attend the ENA. Part of the reason is cost — it can be cheaper to fly to the United States than within Europe, and registration fees are lower. The US meeting has an additional, nonmonetary advantage: it has become a global bazaar for making professional contacts.

In spite of apathy towards the ENA, Europeans still attend their own national conferences in droves. By fulfilling the needs of young postdoctoral researchers to meet prospective employers among their own scientific community, France's Société de Neurosciences and Germany's Neurobiologen-tagung, for example, attract 1,000 or so scientists for meetings in their native language. According to Walter Zieglgänsberger, who organized the Munich meeting, the barrier that ENA must cross is more cultural than linguistic. "People still don't think European", he says, "but in America, they think American".

The ENA also wants to lower the cost of its meetings by replacing professional organizers with local ones and by seeking corporate and philanthropic contributions. Much hope is pinned on the European Commission's plans to support pan-European neuroscience, under the 'Decade of the Brain' initiative. Although not yet accepted by the Commission, many expect this to be the most likely source of the ENA's salvation.

The ENA also realizes that it may need to change its own organizational structure. The association may work more effectively as a federation of successful national societies, most of which want to maintain their own identities, rather than as an organization that chooses its leaders without regard for nationality.

A.A.

First meeting of commission gives Massey a boost in plotting viable future for NSF

Washington. In creating the Commission on the Future of the US National Science Foundation (NSF), the foundation's director, Walter Massey, was seeking help in balancing a congressional directive to use science to strengthen the nation's economy against the foundation's traditional role of supporting academic research. Judging by last week's first meeting of the commission, Massey stands a good chance of walking that fine line.

The commission faces a tall order: to deliver a report by 20 November to the National Science Board, NSF's governing body, setting out principles for NSF to follow in maintaining its relevance into the twenty-first century. The timetable was determined by the November presidential election, although its observations will be included in a strategic plan that NSF has been working on since January.

Gathered at NSF headquarters barely a week after its 15 members — prominent university administrators, researchers and industrial leaders — were selected, the commission not surprisingly spent the first half of its half-day meeting sorting out its task. Although Massey asked the science board to form the commission less than two weeks after the US Senate passed an appropriations bill calling on NSF "to take a more activist role" in transforming the results of basic research into commercial products, NSF officials insist that the commission is not being asked to respond to current events. What is needed, they say, is a thoughtful look into the future by people familiar with the work of the foundation but not dependent on it.

The stakes are high. Rank-and-file university scientists are concerned that NSF may turn its back on investigator-initiated research in favour of directed programmes catering to industrial needs. The result, they say, could be an irreversible decline in an educational system that is recognized as the best in the world. At the same time, politicians are clamouring for a higher rate of return on federal research dollars and looking for high-technology products that will reduce the country's trade deficit.

The discussion at the meeting reflected that ambivalence. No sooner did one member put forward an expansive view of NSF's role, stressing the need for university and industrial scientists to work closely together, than another member reminded the commission of the foundation's basic mission to support academic research and train the next generation of scientists. Of course, some of

the sparring may have been for effect. After an invited speaker, John McTague of Ford Motor Company, described what industry expects from NSF as part of a presentation on the forces shaping industrial research, Marye Anne Foxe, a chemist at the University of Texas at Austin, responded with a quotation from an influential 1945 report by Vannevar Bush entitled *Science, The Endless Frontier* that led to the creation of NSF.



Co-chairs William Danforth, left, and Robert Galvin

"The simplest and most effective way in which the government can strengthen industrial research", Bush wrote, "is to support basic research and to develop scientific talent". When Foxe asked if the commission believed that Bush's words must now be altered to fit today's world, McTague went one better by replying, "I'd engrave them in gold on the walls of the foundation".

But words alone are not enough to clarify NSF's mission. Although NSF officials say

that they do not expect any recommendations from the commission to influence the proposed 1994 budget to be presented to Congress in January, researchers will naturally be looking out for signs of a shift. Similarly, commission members say that they do not wish to labour in vain.

"I would hope that our impact would appear in the federal programs for the diffusion of technology", says William Danforth, chancellor of Washington University in St Louis, Missouri, and cochair of the commission. "Otherwise, we're wasting our time."

It is not at all clear which federal agency should be given the task of ensuring that research is more efficiently turned into products. Commission member Lewis Branscomb of Harvard University says that existing programmes within the departments of Commerce, Defense and Energy do not seem up to the job and that individual agencies with large research portfolios have missions linked to specific sectors of the economy. Branscomb and others are concerned that the government may turn to NSF as a last resort and give it an assignment too large to handle.

The key to Massey's tightrope-walking strategy may lie in words from St Augustine as translated by Harold Shapiro, president of Princeton University, who spoke to the commission about changes occurring within US universities. "Not new, but in a new way", St Augustine said about how to teach the Scriptures. Massey may well be looking for the same formula at NSF.

Jeffrey Mervis

User fees advance in Congress

Washington. User fees paid by drug companies to supplement the budget of the US Food and Drug Administration (FDA) inched closer to becoming a reality last week after winning the approval of an important congressional committee in the House of Representatives. The proposal, the only one of many introduced since the mid-1980s to have won the support of industry, Congress and the president, would allow the cash-starved FDA to recruit 600 more staff to clear the backlog of new drug applications awaiting review by FDA and expedite the drug review and approval process (see *Nature* 358, 616; 1992).

Co-sponsored by Representatives John Dingell (Democrat, Michigan) and Henry Waxman (Democrat, California), the pro-

posal would generate about \$330 million during the next five years through three types of user fees — a one-time payment for new drug applications and annual fees on all approved prescription drugs on the market and on manufacturing establishments. David Kessler, the FDA commissioner, has said that the fees would enable the agency to eliminate its backlog of new drug applications within two years.

Although the bill cuts in half the fees charged to smaller biotechnology companies (those with fewer than 500 employees and no FDA-approved products), those companies are pushing for an exemption for start-up companies with little or no revenue from sales.

Diane Gershon

New rules in Britain make it harder for older scientists to use animals

London. The ability of older scientists in Britain to carry out research using animals has been greatly restricted under a new version of the rules governing such research.

The changes shorten the length of a project licence held by a researcher over the age of 65 and effectively prevent anyone older than 70 from holding a project licence outright. Personal licences, which allow researchers to work with live animals and are usually reviewed every five years, will have to be reviewed annually for those over 70.

The changes are a response to the Feldberg case (see *Nature* 345, 190; 1990) in which the work of 89-year-old Wilhelm Feldberg, then of the Medical Research Council's National Institute for Medical Research, was filmed by animal rights activists and then publicized by the national press. Feldberg was studying the effect of heat on blood sugar levels in rabbits. An inquiry found that Feldberg had breached the Animals (Scientific Procedures) Act of 1986; the incident showed how it was possible for an eminent retired scientist to drift outside the support and control structures set up by the act.

The media exposure and the intention of the act to foster openness led the Animals Procedures Committee, the independent statutory body which oversees the legislation and which comes under the Home Office, to alter the rules for retired scientists. Although the act does not allow researchers

of a certain age to be refused a project licence, the committee has adopted administrative changes that will make it very difficult for a researcher over 70 to hold one. Under the new rules, researchers over 65 can obtain or renew licences only up to the time they are 70.

The easiest solution for researchers caught in this age trap is to have a younger colleague hold the project licence, which should theoretically bring the work back inside the scrutiny of the act.

It is unlikely that these new rules would pass muster in the United States, where discrimination is prohibited on the basis of age in hiring, firing and assigning of duties. Only work that affects public safety, such as firefighting, is exempt. Because animal research has no such impact, says Charles Sabatino, assistant director of the American Bar Association's Commission on Legal Problems of the Elderly, the British policy would probably be illegal in the United States. "It sounds more like a problem with people going off and being independent than with being older," Sabatino says about the new rule.

Although members of the committee concede that only time will tell if the changes are discriminatory, they were felt to be necessary. Not only did the Feldberg case make clear a weakness in the legislation, but it also showed the price of getting caught out.

The annual report of the Animal Procedures Committee also expresses concern over the number of infringements that occur because researchers fail to understand the requirements of the act. As a result, the committee has decided to make formal training a prerequisite before researchers can apply for licences. This change is expected to be implemented by the autumn of 1993 for personal licences and one year later for project licences. The training will cover general matters contained in the act, such as assessing the benefit gained for suffering involved in an experiment and the importance of alternatives to experimental animals, plus the proper conduct of the specific procedures that the licence holder will be doing.

Ian Mundell

US rejects French request to reopen AIDS patent deal

Washington & Paris. The long-running battle over the joint US-French patent for the AIDS blood test reached an official stalemate last week when US officials blocked a request by their French counterparts for an increased share of the patent royalties.

Speaking at the biannual meeting of the foundation that administers the annual royalties, officials from the Institut Pasteur proposed a change in the agreement to give France all the royalties now divided equally between the two countries (the majority of the \$8 million earned annually from the patent) on the grounds that the French deserve most of the credit for discovering the virus. But the four US officials on the eight-member foundation, including Robert Gallo, who is credited in the agreement as the co-discoverer of the virus, and Bernadine Healy, the director of the US National Institutes of Health, voted against the proposal and then walked out. Six votes are needed before any change can be made.

Raymond Dedonder, a member of the French delegation, says he was not surprised; at a previous meeting between the French ambassador and Louis Sullivan, the director of the US Department of Health and Human Services (HHS), Sullivan had warned the French that there "was nothing to consider". Earlier this year, HHS commissioned an independent legal review which found no merit to the French claims.

Dedonder said that no further attempt to reopen the issue would be made until after the US elections in November.

Christopher Anderson & Declan Butler

Worthy proposals get second life

London. A publishing venture begun this week tells British companies about research proposals that, although highly rated, have failed to win funding from the Science and Engineering Research Council (SERC). But scientists whose work is in the first edition of Hydro Oakland's *Research Bulletin* would prefer SERC to fund more of the most worthy projects rather than simply to pass them along to industry.

Under the scheme, SERC will give Hydro Oakland the abstracts of the proposal and the name of the principal researcher who submitted it at the end of each round of grants. The size of the request will remain confidential, however, and researchers whose proposals are listed will be allowed to resubmit their ideas to SERC.

A pilot issue containing around 140 entries in chemistry and process engineering was published this week and will be given free to interested companies. Once established, the bulletins are expected to support themselves through subscriptions, with Oakland needing 200 to break even. SERC

receives no money for the information it supplies. The idea and funding for the project comes from the British arm of Norsk Hydro, Norway's largest industrial company.

The difficulty in getting money for even the most worthy proposals frustrates many British researchers, and the majority of those with entries in the first bulletin welcome the extra exposure. Although on average 70 per cent of alpha-rated proposals are funded, this percentage varies considerably according to discipline. Last year, for example, 90 per cent of the alpha-rated proposals in process engineering were funded but only 22 per cent in chemistry.

However, the prospects for funding are sufficiently poor that most researchers believe they routinely explore every avenue for industrial collaboration through industrial liaison offices or personal networks. Although SERC is supposed to ensure that university research is exploited by industry, most researchers do not see that as its most important function.

Ian Mundell

NIH cDNA patent rejected; backers want to amend law

Washington. The US Patent Office has rejected a controversial application by the National Institutes of Health (NIH) for patents on more than 2,000 cDNA sequences. Although NIH is expected to appeal against the decision, the interim ruling could set a precedent that would ban patents on genes that share even tiny sections with already published sequences.

The ruling on 20 August finds the NIH sequences unpatentable on the grounds that they are 'obvious': many of the sequences contain small fragments that the patent office was able to find in other, already published, sequences. Once part of a sequence has been published, according to the patent office, it is a trivial matter to get a larger sequence or the entire gene.

Both NIH and Congress are expected to attack that decision on the grounds that, if allowed to stand, it would make impossible a patent for any gene containing sequences already patented. The NIH application, for example, covers fragments of human genes, several hundred bases long, whose function is unknown (see *Nature* 353, 485; 1991). Even if the patent office were eventually to grant that application, last month's ruling would deny a patent to those who had sequenced the full genes and found their function. That would effectively discourage many biotechnology companies from trying to find base products on those genes.

The patent office also ruled that the claimed sequences lack legal novelty because they were derived from publicly available cDNA libraries. In congressional testimony early this week, Bernadine Healy, the NIH director, also challenged that interpretation. "Taken to its logical extension, [the patent office reasoning] would deny novelty to other products isolated from expected sources of biomolecules, such as blood saliva or tissues", she argued.

At the same hearing, former NIH geneticist Craig Venter proposed a legislative solution to the dilemma posed by the patent office decision. Venter, who discovered the sequences last year but has since left NIH to start a new private research institute to pursue cDNA sequencing, suggests amending US patent laws to allow patents on sequences containing previously patented or published fragments. Senator Pete Domenici (Republican, New Mexico) intends to introduce the measure early next year.

Venter says that his change would not affect the status of his NIH application but would apply to subsequent filings. Changes to the patent code are not retroactive.

NIH officials say they were particularly disappointed with the ruling because it contains little discussion of the merits of gene patents and takes instead an extreme view on what might be called the inventiveness of the discoveries. If, the patent office argued, the cDNA sequences (which represent fragments of expressed genes of unknown function) contain base sequences that have already been published, then they fail the obviousness test; small fragments of the published sequences (from any species) could be used as a probe to search human DNA for a match.

NIH is partly to blame for this conclusion. In its application, NIH defines the sequences as both the full 400–500 bases its researchers found for each gene and any 15-base fragment—known as a 15mer—of the larger sequences. Fifteen has traditionally been regarded as the minimum number of bases required to probe for a unique gene in unknown genetic material.

Using that yardstick, the patent examiners selected 15mers at random from some 20 sequences in the NIH application and searched through databases of published sequences. After comparing the 15mers with 300–400 random sequences, they found a number of matches, including one to a mouse gene. That, they concluded, was enough to undermine NIH's case. "It would be obvious for one ordinarily skilled in the art" to take one of the published 15mers and use it to probe for the NIH sequence in which it appears, the patent office concluded in its 30-page interim ruling.

NIH's mistake was not in trying to pass off already published mouse genes as human brain cDNA but rather in assuming that a 15mer would uniquely identify a gene. Despite the convention that 15 bases are enough for a unique probe, most researchers now believe that 30, or even 50, bases may be necessary to avoid the sort of spurious matches found by the patent office.

NIH has at least three months to file a revised application with the patent office. Although officials at its parent agency, the Department of Health and Human Services, would like NIH to withdraw its application and end the controversy, NIH patent officials are optimistic that their arguments will eventually carry the day. If the patent is indeed granted, Healy promised that NIH would not enforce its rights to any partial sequences of unknown function. Complete genes whose function has been determined are a different matter, she said, but NIH has not yet decided at what point to set that threshold.

Christopher Anderson

Emphasis on research blamed for problems at US universities

Washington. A new congressional report that blames the declining quality of public undergraduate education and soaring tuition fees on a preoccupation with research is viewed by university administrators and faculty as an uninformed attack on the role of research in education.

The report into the rising cost of a college education, prepared by the Select Committee on Children, Youth and Families of the US House of Representatives and released last week, accuses four-year state universities of emphasizing research for self-aggrandisement at the expense of undergraduate education. When professors spend too much time in the laboratory, according to the report, the results are large classes and a reliance on teaching assistants. The report contains data from a survey showing that, although faculty members teach for fewer hours than they did 30 years ago, few spend this extra time doing research.

The belief that 'quality' education is synonymous with high-powered research has also contributed to higher tuition fees, according to the report. Universities must hire more professors or teaching assistants to fill the gap left by faculty working at the bench, and their failure to serve as student advisers and carry out "other duties traditionally associated with the teaching profession" has forced universities to hire additional staff.

Although the report addresses some critical issues, such as the increasing use of teaching assistants, university officials say that its statistics are misleading and its conclusions naive. Statistics that lump together faculty members from disparate schools give an inaccurate picture of how professors spend their time, and recent sharp increases in tuition fees stem not from the neglect of teaching but from growing enrolments and insufficient funding from the state.

The report portrays research as an activity that public universities pursue to gain a cachet as elite institutions. But students who cannot afford private schools can gain research experience only at public universities, and research opportunities also help to attract the best faculty members.

University administrators are also unhappy that, at the congressional hearing on 14 September coinciding with the release of the report, the sole witnesses were two former academic officials and two students from large state universities whose statements echoed the arguments in the report. A congressional staff member who worked on the report explained the imbalance by saying that the academic community has had plenty of chances to testify "and the other side has not".

Traci Watson

Alberts emphasizes education as nominee to head NAS

Washington. With one foot in the laboratory and the other in an elementary school classroom, biochemist Bruce Alberts is ready to take a cross-country leap into the presidency of the National Academy of Sciences.

Alberts, an American Cancer Society research professor at the University of California, San Francisco (UCSF), has been nominated by the academy's governing Council to succeed Frank Press, who is stepping down in July at the end of his second five-year term. There will be a postal vote in December by the academy's 1,640 members and no other names have been put forward by the council.

If elected, Alberts will end the academy's recent tradition of being led by a well-known science administrator. (Press was science adviser to President Jimmy Carter, and his predecessor, Frederick Seitz, was president of the Rockefeller University.) "They said that they wanted a working scientist", Alberts says of the nominating committee that pursued him for several months. "And they told me that I could still do science, if not at the bench, then at least by writing review articles and working on the third edition of my textbook [*The Molecular Biology of the Cell*, first published in 1983]".

The 54-year-old Alberts has been a pioneer in studying DNA replication through his work on bacteriophage T4, and he remains active in that area and in studying cell differentiation in the cytoskeleton. He directs a 12-person laboratory that receives substantial funding from the US National Institutes of Health, and he has reluctantly

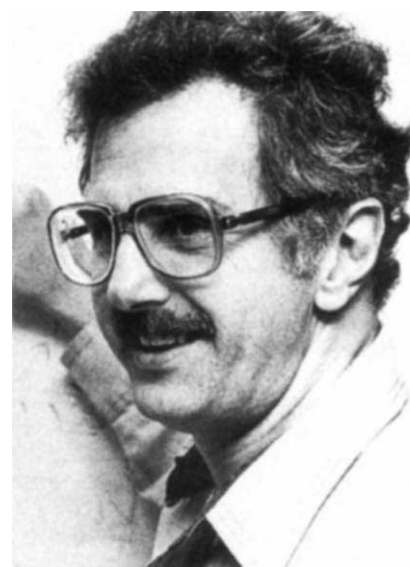
decided to close the laboratory over the next two years because of the impossibility of trying to run it from a distance of 3,000 miles. But he does not rule out a return.

"Running a lab is a full-time job", he says. "So I guess that I'll be doing this [serving as NAS president] for a while rather than trying to cure cancer. But right now my preference is to serve one term and come back out here. I love San Francisco, and I'm not going to sell my house. I don't plan on becoming a Washington type."

Equally important to Alberts as his science are his efforts to improve the quality of science education in US elementary and secondary schools. He is heavily involved in a programme within the San Francisco public schools, and he has written extensively about the need for scientists to help public school teachers to rekindle a sense of wonder in children about how the world works. Alberts says he accepted the NAS nomination only after deciding he could make a contribution in Washington.

"Leadership in science matters", he says. "Frank Press has been moving the academy in the right direction, but [science education] needs much more attention than it gets from the community. We need to motivate people and give them incentives to get involved. There's a lot of goodwill and idealism out there, but you need to show people how they can make a difference."

Last December, Alberts helped to organize a two-day workshop before the annual meeting of the American Society for Cell Biology for scientists and public school teach-



Alberts is leaving his heart in San Francisco.

ers; "I was at UCSF for ten years before I met a high school science teacher", he confesses. But public schools need more than moral support: they also need materials, time and continuing access to the latest scientific information, and Alberts hopes to raise the money to make those things possible.

The president of the National Academy of Sciences wears many hats, including that of fund-raiser, public spokesman for the scientific community and source of impartial information for the federal government. Alberts knows that he is less experienced in those areas than some of his predecessors, but he is not fazed by the challenge.

"Nobody has enough knowledge to take this job," he says. "But the academy is more than one person, and there are a lot of people who can help me to do mine."

Jeffrey Mervis

OST rows with tide in report on intellectual property

London. The first report by the British government's new Office of Science and Technology (OST) emphasizes the importance of finding applications for whatever research the government is willing to sponsor. Both the topic — intellectual property rights from publicly funded research — and the recommendations of the report reinforce the belief of the science minister, William Waldegrave, that because there will be no new money next year for British science, any changes must be self-supporting.

The report* is the first to be produced by the Office of Science and Technology (OST), created after April's general election. The office is responsible for producing a white paper (policy document) next year on science.

Most interesting as an indicator for the future is the report's concern with changing the scientific culture. It says that students

should be made aware of issues relating to intellectual property and that its exploitation should be part of the rewards and incentives for scientists.

In many instances, the report reflects what is already going on. For example, the Medical Research Council (MRC) has a system widely admired for distributing the material rewards of intellectual property to the individual, the institution and the council, and it has also encouraged the recognition of patents in assessing achievement. The Agricultural and Food Research Council is in the process of adopting a similar reward system. By sanctioning such trends, the OST has assured its report a warm welcome and increased the chances that what it recommends will come to pass.

How useful this approach will be when the government wants to make changes that go against the grain remains to be seen. One

of its more contentious proposals is for research councils to give greater weight in assessing research proposals to innovative applications of existing knowledge.

"The government wants to be seen to be even-handed, and it thinks that if it could just hit on the right magic formula, that would be applicable across the science base. But the process does not work the same way in every area," says David Owen, the MRC's director of industrial collaboration and licensing. Pharmaceutical companies, the chief market for the MRC's intellectual property, feel they are better placed to carry out commercial innovation using existing knowledge and instead look to the MRC for basic research. "We are good at one, they are good at the other", he says. **Ian Mundell**

* *Intellectual property in the public sector research base* (£10.50 from HMSO, PO Box 276, London SW8 5DT, tel. 071-873-9090).

Rewriting the history of science

SIR — In his review of Mary Midgley's book *Science as Salvation* (*Nature* 357, 550; 1992), Stuart Sutherland overlooks Midgley's attempts to rewrite history. According to her, "Descartes's distinctive move, which made room for Galileo and modern physics, was not his scepticism, but his finding a way out of that scepticism by a radical act of trust in scientific reason" (p.124). Not so. Galileo (1564–1642) was 32 years senior to Descartes (1596–1650). By the time Descartes was 20, Galileo was already under investigation by the Inquisition for his scientific reasoning¹.

Moreover, Midgley writes that "mechanistic scientists who fill the rest of our pantheon rejected Kepler's view fiercely as superstitious. In particular Galileo, who might have been expected to welcome Kepler's support for the Copernican system, simply ignored it" (pp 82–83). Kepler in fact gave two copies of his first book to Paul Hamberger to leave with astronomers in Italy. Both were given to Galileo, who on 4 August 1597 wrote in haste to thank Kepler, explaining that he had had the book only a matter of hours, without time to study it, although the return of Hamberger to Germany made it imperative for him to write at once. Indeed, Galileo rejoiced to find that Kepler supported Copernicus, whose view Galileo had now held for a number of years².

Midgley fails to appreciate that the reason why Galileo did not talk openly about new ideas in favour of Copernicanism was that he was intimidated by the Church's condemnation of Copernicus. Kepler's and Copernicus's works were on the Jesuit Index; they could not legally circulate within the ambit of the Church. Galileo had already in 1616 had a friendly 'fireside chat' with Cardinal Bellarmine, and was effectively silenced (he was forced by the Inquisition to recant his views and sentenced to life imprisonment in 1633). By contrast, Kepler was a Protestant in Protestant Germany and free to speak, while Descartes, although having the protection of the French Court, migrated first to Holland and then to Protestant Sweden.

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1. Redondi, P. *Galileo: Heretic* (Lane, London, 1988).
2. Drake, S. *Galileo at Work: His Scientific Biography* (University of Chicago Press, 1978).

SIR — I understand Steven Rose's annoyance (*Nature* 359, 10; 1992) at Stuart Sutherland's casually terming J. D. Bernal and J. B. S. Haldane as "crackpots". But I am annoyed with

Rose for quoting Sutherland flagrantly out of context.

Sutherland wrote that Bernal and Haldane "became crackpots when they left their laboratories" to engage in "armchair utterances". Their grasp of complex political and social problems was often (not always) more tenuous than their grip on the physical and life sciences, a manifestation many other gifted scientists have displayed from time to time. What Sutherland is in fact saying is that, brilliant as Bernal and Haldane were in their fields of expertise,

once out of them they were capable of writing just as stupidly as the rest of us, thus allowing individuals such as Mary Midgley to pounce on their sillier remarks and use these as sticks with which to beat science in her (and others') crusade against science's "soullessness" and "amorality".

If Rose then (rightly) claims that Midgley *et al.* are being less than honest with their selected quotations, he should bear this in mind to his own advantage.

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Brown's observations confirmed

SIR — The recent claims that Robert Brown could not have observed brownian movement can now be laid to rest. I have demonstrated videomicrographs of the phenomenon, seen through Brown's own microscope, at Inter Micro (Chicago, Illinois, July 1992). The recordings reveal the clarity with which Brown observed the phenomenon that now bears his name.

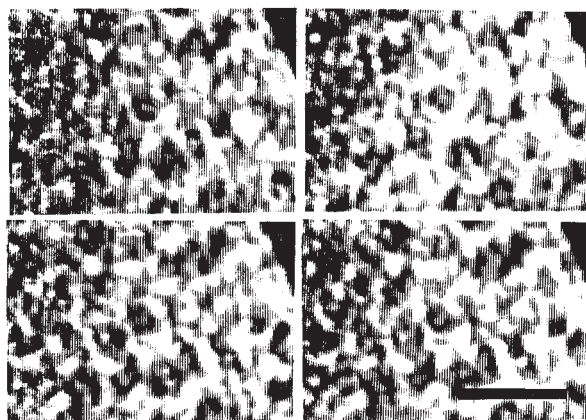
Your correspondent Cadée¹ is taken to task by Deutsch² for a slight misquotation in his letter. Deutsch com-

Brown's privately printed account⁴.

The authority now cited by Deutsch⁵ produced an interesting account, although it perpetrated the widespread belief that the simple microscope was not capable of generating images of sufficient resolution. Perrin writes that the phenomenon was described "very shortly after the discovery of the achromatic objective". This comment is misleading.

As Brown makes clear, his observations were made using the simple (single

Four frame enlargements from video imaging of brownian movement. The test specimen is suspended globules in milk, and the scale bar represents 10 μm . Frame separation 0.5 seconds, and the high-power lens from Brown's microscope is calibrated at a magnification of $\times 170$. It resolves particulates of 1.3 μm diameter. The microscope is in the collection of the Linnean Society.



lens) microscope. He had brief recourse to an early achromatic compound system, but soon returned to the single lens³. The "pseudo-brownian movement" postulated by Deutsch is certainly not recognized by Perrin, or by other major workers in the field. I am aware of no evidence that it exists.

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As Brown makes perfectly plain, his observations were of the intracellular granules within the pollen cells, and not of the entire grains themselves³. Furthermore, Brown took great pains to avoid external perturbations and was clearly aware of the problems that might be caused by currents induced by such factors. Each of Deutsch's objections can be faulted by a careful consultation of

1. Cadée, G. C. *Nature*, 354, 180 (1991).
2. Deutsch, D. H. *Nature*, 357, 354 (1992).
3. Brown, R. A brief account of microscopical observations made in the months of June, July and August 1827 on the particles contained in the pollen of plants and Additional remarks on active molecules (London, 1828).
4. Ford, J. *The Microscope* 39 (3 & 4), 161–173 (1991).
5. Perrin, J. *Brownian Movement and Molecular Reality* (London, 1910).

Remedies for brain disorders

SIR — The dismay of several distinguished scientists¹ at reading your leading article sceptical of the applications of our new knowledge to human disease² is understandable. *Nature* is, however, essentially correct in saying that we are still quite far from appropriate and effective solutions to the problems created by various severe neurological and mental illnesses. At best, we are now at the end of the beginning, thanks to the development of sophisticated tools that may be useful for the understanding of disease processes and eventually for the development of better remedies. We should not repeat the costly mistakes made in the 'war on cancer', when some important but limited successes led to a premature announcement of the beginning of 'victory'.

In the case of the major psychoses, there is no scientific agreement whatever on aetiopathogenesis, let alone on some critical nosographic problems. Specifically, the statistical distribution of symptoms and of the varied course of disease in large groups of psychiatric patients does not show the peaks and valleys essential to support the hypothesis of distinct pathological conditions (categorical models). Rather, the data speak of a continuum from 'pure' bipolar disease and psychotic depression to 'pure' schizophrenia (dimensional model)³. Yet most hard research of a biomedical kind takes one or other categorical model as a basic assumption; such practices may be unavoidable, but the limitations and biases should be more explicitly declared.

The available therapies serve almost exclusively to control symptoms, undoubtedly an important goal in certain circumstances. But the cost is often high. With the neuroleptic drugs in particular, as well as the high risk of irreversible neurological damage causing tardive dyskinesias, there is growing concern about the contribution that treatment may make to the development of organic dementia⁴ and the marked unpleasantness of the drugged stage in a substantial number of the treated patients (neuroleptic dysphoria)⁵. The last phenomenon is clearly supported by reports that some neuroleptics evoke strong aversion in animal studies⁶, while some authors support the view that there may be a link between neuroleptic use and the development of a chronic dysphoria-depression syndrome⁷. In fact, abating the unpleasantness of psychiatric therapies is one of the goals proposed by the World Health Organisation⁸.

I gradually gained a painful and sobering awareness of these and other related problems thanks to several years of close

contact with practising neurologists and psychiatrists, clinical psychologists, nurses, social workers and some of their more difficult patients — an exercise that may be strongly recommended to anyone doing basic research in the neural and/or the behavioural sciences. I agree that some of the statements in *Nature*¹ may have been a bit too strong. In our culture, however, this is the only tool we have to hammer an important point; therefore we must appreciate, rather than resent, your decision to break the glass on the alarm.

Giorgio Bignami

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1. *Nature* **358**, 184 (1992).
2. *Nature* **357**, 348 (1992).
3. Jablensky, A. in *Epidemiological Impact of Psychotropic Drugs* (eds Tognoni, G., Bellantuono, C. & Lader, M.) 71–97 (Elsevier/North-Holland, Amsterdam, 1981).
4. Breggin, P. R. *J. Mind Behav.* **11**, 425–464 (1990).
5. Emerich, D. F. & Sanberg, P. R. *Biol. Psychiat.* **29**, 201–203 (1991).
6. Bignami, G. *Biol. Psychiat.* **30**, 844–845 (1991).
7. Harrow, M., Fichtner, C. G., Grossman, L. S., Yonan, C. A. & Sands, J. *Biol. Psychiat.* **30**, 845–847 (1991).
8. Sartorius, N. *Croat. Med. J.* **33**, 3–8 (1992).

Genetic influence

SIR — Christopher Anderson (*Nature* **358**, 357; 1992) accurately describes the controversy surrounding the National Institutes of Health (NIH) funding freeze for the forthcoming genetics and crime conference, but says little about the conference itself. As its organizer, I would like to fill that gap. The conference will offer a public forum for examining the direction and impact of research concerning genetic influences on criminal behaviour, and for debating the validity, interpretation and applications of that research. Far from endorsing the assumptions or goals of research programmes in human behavioural genetics, the conference will subject them to close critical scrutiny.

The conference will address many of the questions its opponents are now raising. Do genetic explanations of behaviour undermine or refine environmental explanations? Does genetic research focus on some kinds of crime to the exclusion of others? Will that research divert attention from social causes of crime? How can genetic factors explain socially defined behaviour? What uses will be made by the criminal and juvenile justice systems of the claims of genetic influence and genetic predisposition likely to emerge from current research? And how will those claims affect public perceptions and broader social policy? For those concerned about the

public oversight and policy implications of scientific research, this conference presents an opportunity, not a threat.

According to Anderson, Dr Bernadine Healy, director of NIH, claimed she could not defend the conference against public criticism for want of a "ringing endorsement" by its reviewers. In fact, the conference did receive a ringing endorsement from its reviewers: the study section described the proposal's analysis of the issues as "superb" and the array of speakers as "impressive". It concluded that the conference was "likely to be considered a landmark in the field". Healy's selective contact with members of the study section represents a further subversion of the NIH review process.

I am concerned about the way in which this conference has been perceived by some members of the black and activist communities, and I was consulting the National Center for Human Genome Research to modify the brochure and agenda well before Healy imposed her illegal freeze. Her politically motivated response has hampered our efforts to address important issues about the social impact of behavioural genetic research.

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Adders multiply

SIR — Madsen *et al.* (*Nature* **355**, 440–441; 1992) ask "Why do female adders copulate so frequently?" Reproductive success in the adder depends strongly upon the substrate, and the abnormally high frequency of copulation that the authors observed may in fact merely reflect the lack of suitable substrate in the Swedish meadowlands where the observations were made. The importance of this variable is related in the following biblical legend.

When Noah's ark landed on Ararat, he ordered the departing animals, in the name of the Lord, to be fruitful and multiply. All his charges were more than happy to comply, save for the pair of adders, who reminded Noah that since they were mere adders, he could not very well expect them to multiply. Not one to take no for an answer, Noah took the adders to a vacant room, placed them on a table, and told them he was locking them in until they obeyed the Lord's command to multiply. When Noah next looked in on them, they had indeed managed to multiply, for he had wisely placed them on a log table.

David C. Jolly

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The search for dark matter (contd)

An article with an arresting title puts forward a novel way of resolving the question whether the matter that holds the Universe together is 'cold' or 'hot' by showing that one kind of particle might fill both roles.

ONE way of making a research article catch the eye is the well-worn trick of uniting in the title a number of disparate concepts, raising in the potential reader's mind the question of how a single article can deal with such a variety of things. Perhaps the best example is the title of the late Jacques Monod's book *Chance and Necessity*, but here is another, the title of a research article that reads "Bose condensates, Big Bang nucleosynthesis and cosmological decay of a 17 keV neutrino". The author, Jes Madsen from the University of Aarhus, must be sure that hardly any of the readers of *Physical Review Letters* for 27 July will have failed to pause at page 571.

What Madsen has to say is as intriguing as the title. The underlying question is whether neutrinos may account for the cosmological dark matter. The 17 keV neutrino is the object for which evidence was provided by J. J. Simpson in 1985, but which has not yet been generally accepted as one of the menagerie of particles. The 17 keV refers to the supposed mass of the neutrino, which works out at about 3 per cent of the electron mass. So much is clear from the title, but where do "Bose condensation" and "Big Bang nucleosynthesis" fit in?

Bose condensation evidently refers to the behaviour of a collection of bosons (particles with zero or integral spin in the quantum sense). The superfluid component of superfluid helium, for example, is a Bose condensate. What can be the connection with the 17 keV neutrino, which is also supposed to be the neutrino corresponding to the τ -electron? If the neutrino exists, there is some evidence that it must be unstable, and that one of the products of the decay is the more familiar electron neutrino, called ν_e . But in that case, there must be another particle to carry away some of the energy, and because the neutrinos are both fermions (particles with spin half), the missing particle must be a boson.

Indeed, the argument is more general than that. If the 17 keV neutrino is supposed to decay in the simplest possible way into two particles, one must be a fermion (to account for the half-integer spin of the original neutrino), not necessarily the electron neutrino, and the other must be a boson. And there will also be an antineutrino corresponding to the 17 keV particle and then antiparticles corresponding to each of the two decay products. Madsen reminds his readers that the inverse of the decay process will also be possible.

If these 17 keV neutrinos are real, and if

they have something to do with dark matter, there must be quite a number of them and, being fermions, their behaviour will be governed by the Pauli exclusion principle (that no more than two may be in the same quantum state), which in turn implies that if the sea of neutrinos is in thermodynamic equilibrium with itself, the energy of the particles (or, more helpfully, their momentum) being distributed according to Fermi-Dirac statistics.

Equilibrium, of course, implies some temperature, which is most conveniently measured in electron-volts (avoiding the use of Boltzmann's constant). Madsen is chiefly concerned with temperatures such those in the early stages of the Big Bang, measured perhaps in MeV. Equilibrium also implies some means of interaction between the particles themselves or with matter of some other kind, supposed to be done through the weak nuclear interaction (and which would have been efficient in the early Big Bang Universe). If the sea of 17 keV neutrinos is indeed a Fermi-Dirac distribution, the number density of neutrinos with a particular momentum will be essentially independent of momentum up to a value comparable with the temperature (in energy units), above which the density will fall off with increasing momentum. (The cut-off would be abrupt if the temperature were zero.)

It is then straightforward (if complicated) to calculate the number-density of the decay products as a function of momentum, after making an assumption about the decay half-life. (The complication is the need to transform from the rest-frame of the particle, in which each decay product will have the same momentum, to the frame of the real Universe.) And this is where the interest lies. Being a fermion and a boson, and given that the weak nuclear interaction should bring them into equilibrium among themselves, the decay products should themselves satisfy Fermi-Dirac and Bose-Einstein statistics respectively.

Several unexpected things follow from this, not the least of which is that the effective speed of the decay of the 17 keV neutrinos is constrained by the inverse reaction. Even if the half-life of the 17 keV neutrino in isolation is as little as a tenth of a second, the inverse reaction between the decay products to recreate 17 keV neutrinos will increase the effective half-life to about 100,000 seconds — rather more than a day.

The bosons involved in this process, whatever they may be, raise other possibilities. With momenta distributed by Bose-

Einstein statistics (whose characteristic is that low or even zero-momentum states are occupied preferentially), if the bosons have a small mass, a fraction of them may form a Bose condensate, which means that they will move as if they were a collective whole. Just what fraction of bosons would condense in this way into the lowest energy state depends on the temperature and the lifetime, but Madsen argues that as much as a third of the decay bosons might be thus contained.

That immediately provides a new way of looking at the dark-matter conundrum: if a fraction of the bosons hang together collectively and if each of them has a small amount of mass, they will behave individually as particles of 'cold' dark matter with negligible kinetic energy. But the remainder will be energetic, and will behave as if they were 'hot' dark matter. Cosmology, in the aftermath of the COBE detection of fluctuations and recent surveys revealing more large-scale galaxy clustering than expected, may require quantities of both hot and cold dark matter. So one intriguing feature of Madsen's argument is that one kind of particle may account for both the cold and the hot dark matter. All that is left to do, his readers will conclude, is to find that boson.

That accounts for the "Bose condensation" in the title of Madsen's paper. What about the "nucleosynthesis"? In the standard model of the Big Bang, the formation of the light nuclei such as deuterium and ^4He is powerfully influenced by weak interactions with particles such as neutrinos, notably electron neutrinos. The point here is that neutrinos should have appeared on the scene before the ratio of protons and neutrons in the Universe was fixed. But if one of the decay products of the 17 keV neutrinos is indeed the electron neutrino, the energy distribution of those particles will be altered and so will be the yield of the different light nuclei.

Madsen is especially pleased that his outline of what the 17 keV neutrino will do for the early Universe is to admit a somewhat lower abundance of ^4He than in the standard calculation. He notes that estimates of this abundance of ^4He have been falling steadily over the years and that, although the latest estimates are marginally consistent with the standard calculation, the decay of a 17 keV neutrino would allow a lower helium abundance without affecting calculations of the primordial production of deuterium, ^3He and ^7Li , with which the data are more closely in agreement. **John Maddox**

In search of the soluble

John Hardy and Mike Mullan

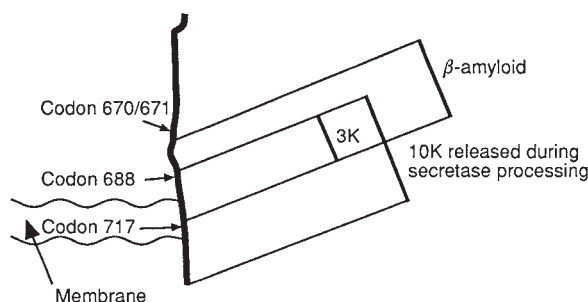
THE identification of mutations in the β -amyloid precursor protein (APP) gene which cause familial, early onset Alzheimer's disease¹⁻⁴ is the strongest evidence that amyloid metabolism is the central event in the pathogenic process⁵⁻⁷ underlying the disease. These rare genetic cases point to the need to identify the key sites of metabolism of the APP molecule at which the balance between pathogenic and nonpathogenic pathways can be influenced. Now, with the reports on pages 322 and 325 of this issue^{8,9} and similar observations from groups led by S. G. Younkin¹⁰ and B. Yankner (personal communication), progress is being made on that front. Together with other papers describing further APP mutations^{4,11}, the new findings provide the beginnings of an integrated theory of the molecular pathogenesis of Alzheimer's disease.

APP is a glycosylated transmembrane protein with a large extracellular domain, a single transmembrane domain and a small cytoplasmic domain. The β -amyloid fragment composes part of the transmembrane domain and a short stub of the extracellular domain¹² (all numbering is given relative to the APP770 transcript: on this scheme, β -amyloid goes from aspartate 672 to threonine 714) (see figure). There seem to be at least two major pathways for APP metabolism. The first, the 'secretase' pathway, cuts within the β -amyloid portion of the molecule before leucine 688 (ref. 13). This cut probably occurs on the cell surface and appears to be reasonably independent of the sequence around the cut site. Instead, it recognizes α -helical conformation at the appropriate distance (12-13 residues) from the membrane¹⁴. The second pathway, the endosomal lysosomal pathway, cuts close to, but probably outside, the β -amyloid fragment. A likely site of cleavage is at lysine 670. This pathway is potentially amyloidogenic as it yields carboxy-terminal fragments containing the intact β -amyloid sequence¹⁵⁻¹⁷.

Nonetheless it has remained difficult to see how these carboxy-terminal fragments could be further processed to become β -amyloid. Essentially, there were two possibilities. Either the carboxy-terminal fragments come out of the membrane and begin to aggregate; under these circumstances, the further proteolysis could be nonspecific extracellular breakdown. Or β -amyloid is cleaved from the carboxy-terminal frag-

ment by a specific process within the membrane. Both of these schemes have problems associated with them. In particular, it is difficult to imagine how transmembrane proteins could come out of membranes, or be cleaved within them.

However, the authors of the two papers in this issue^{8,9} now show that soluble β -amyloid is produced by healthy cells in culture and in human and animal



Mutations in the gene for the β -amyloid precursor protein that are pathogenic in Alzheimer's disease, in relation to normal processing products.

cerebrospinal fluid. Other groups have similar results (ref. 10 and B. Yankner, personal communication). Taken together, these observations suggest that cleavage of β -amyloid must be taking place in the membranes in intact and healthy cells and tissues. The cellular and subcellular location of this process remain unclear, however. Another conundrum is that the soluble peptide is predominantly 40 amino acids long, whereas the main component of β -amyloid in plaques is two to three amino acids longer at the carboxy terminus. This discrepancy is not easy to reconcile with a precursor-product relationship between soluble and plaque amyloid, although it might reflect the occurrence of minor, longer components in the soluble β -amyloid which are selectively deposited.

How do these biochemical observations fit in with the positions and phenotypes of the APP mutations? Eight variant sequences of APP have now been reported (for review see ref. 18), six of which lead to β -amyloid deposition. Besides the three variants at APP717 changing valine to isoleucine, phenylalanine and glycine¹⁻³, and causing Alzheimer's disease, the only other variant which seems invariably to result in disease is a double mutation changing lysine-methionine at APP670/671 to asparagine-leucine. The positions of these mutations are a matter of considerable interest because they juxtapose the sites at which the two cleavages lib-

erating β -amyloid must occur.

A plausible hypothesis would be that the mutations facilitate the APP processing down the β -amyloid generating route(s) and favour this pathway of metabolism over both the secretase pathway and other lysosomal pathways. In this way, marginally more β -amyloid of normal sequence might be formed. Alzheimer's disease in these cases may thus resemble the disease in Down's syndrome in its pathogenesis (overproduction of β -amyloid¹⁹). Besides the four disease-causing mutations, there are two mutations within the β -amyloid sequence which lead to deposition, but to phenotypes different to those characteristic of Alzheimer's disease.

These are the change from glutamate to glycine at APP693, which causes hereditary cerebral haemorrhage with amyloidosis (Dutch) (HCHWA-D)²⁰, and that from alanine to glycine at APP692, which appears to cause a phenotype that resembles Alzheimer's disease in some patients but HCHWA-D in others.

One possible explanation of pathogenesis in cases with these mutations is that the abnormal β -amyloid which is produced is less soluble than normal β -amyloid, and is thus more rapidly deposited at sites where its solubility is exceeded²¹.

What do these findings mean for the diagnosis and treatment of Alzheimer's disease? One might expect that people with the disease would produce more β -amyloid, and that drugs which reduce β -amyloid production might be beneficial: already, however, there are preliminary data which seem to suggest that people with Alzheimer's disease do not necessarily synthesize more β -amyloid¹⁰. Much more work is required before the

- Goate, A. et al. *Nature* **349**, 704-706 (1991).
- Murrell, J., Farlow, M., Ghetti, B. & Benson, M. *Science* **254**, 97-99 (1991).
- Chartier Harlin, M. C. et al. *Nature* **353**, 844-846 (1991).
- Mullan, M. et al. *Nature Genet.* **1**, 345-347 (1992).
- Glenner, G. G. & Murphy, M. A. *J. neurol. Sci.* **94**, 1-28 (1989).
- Selkoe, D. J. *Neuron* **6**, 487-491 (1991).
- Hardy, J. & Allsop, D. *Trends Pharmacol.* **12**, 383-388 (1991).
- Haass, C. et al. *Nature* **359**, 322-325 (1992).
- Seubert, P. et al. *Nature* **359**, 325-327 (1992).
- Shoji, M. et al. *Science* (in the press).
- Hendricks, L. et al. *Nature Genet.* **1**, 218-221 (1992).
- Kang, J. et al. *Nature* **325**, 733-736 (1987).
- Anderson, J. P. et al. *Neurosci. Lett.* **128**, 126-128 (1991).
- Sisodia, S. S. *Proc. natn. Acad. Sci. U.S.A.* **89**, 6075-6079 (1992).
- Estus, S. et al. *Science* **255**, 726-728 (1992).
- Goide, T. E., Estus, S., Younkin, L. H., Selkoe, D. J. & Younkin, S. G. *Science* **255**, 728-730 (1992).
- Haass, C., Koo, E. H., Mellon, A., Hung, A. Y. & Selkoe, D. J. *Nature* **357**, 500-503 (1992).
- Hardy, J. *Nature Genet.* **1**, 233-234 (1992).
- Rumble, B. et al. *New Engl. J. Med.* **320**, 1446-1452 (1989).
- Levy, E. et al. *Science* **248**, 1124-1126 (1990).
- Wisniewski, T., Ghiso, J. & Frangione, B. *Biochem. biophys. Res. Commun.* **179**, 1247-1254 (1991).
- Khachaturian, Z. *Neurobiol. Aging* **13**, 197-198 (1992).

relationships between aetiological factors for the disease and biochemical markers of the disease are elucidated. In particular, the search for further genetic errors altering APP metabolism must continue, and the relative rates of the different metabolic routes for APP in tissues from control subjects, individuals with Down's syndrome and individuals with defined mutations need to be determined.

Finally, working out the relationship between the pathogenic APP derivatives and initiation and progression of the disease will present the most challenging

area of research. Realization of such a synthesis remains a gleam in the eye. But with that, and the use of transfected cell lines and transgenic animals, we would be on the way to achieving the goal of delaying the onset of Alzheimer's disease by five years in five years and by ten years in ten years²². □

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OPTICAL MATERIALS

Fresh start for photonics

Larry R. Dalton

PHOTONICS, the optical analogue of electronics, promises an exciting new age of technology based on optical computing, optical radar, high-speed communication and so on (see figure). But based on materials with nonlinear optical properties (the refractive index or absorbance changes with light intensity or applied fields), the notion has been held back by difficulties in finding examples where these effects are strong enough for practical purposes. One possible way forward is shown by Garito and colleagues on page 309 of this issue¹, who test an idea they propounded previously² that large nonlinearities may be obtained by using molecules in their excited states instead of their ground states. Indeed, they show that the optical nonlinearity of diphenyl-hexatriene is 1,000 times greater in its excited state than in its ground state.

The nonlinear interaction of light with matter can be divided into two categories, resonant and nonresonant. In resonant phenomena, light is absorbed by matter, exciting electrons from a lower to a higher state. In this process, the transmission of light through a material is attenuated and the matter is heated as the result of relaxation processes which

return the electrons to their original or equilibrium states. Typical resonant nonlinear processes include saturable absorption, in which high light intensities overwhelm a material's ability to absorb light, and photoinduced absorption, in which a normally transparent material becomes absorbing at high intensity.

In nonresonant processes, there is a coherent interaction between light and matter which does not involve absorption of energy or population of electronic excited states. As a consequence the index of refraction, n , changes with the intensity of light, I : $n = n_0 + n_2 I$, where n_0 and n_2 are the linear and nonlinear indices of refraction respectively. It is the intensity-dependent beam steering that results from the nonlinear index term that is the basis of many applications such as optical computing.

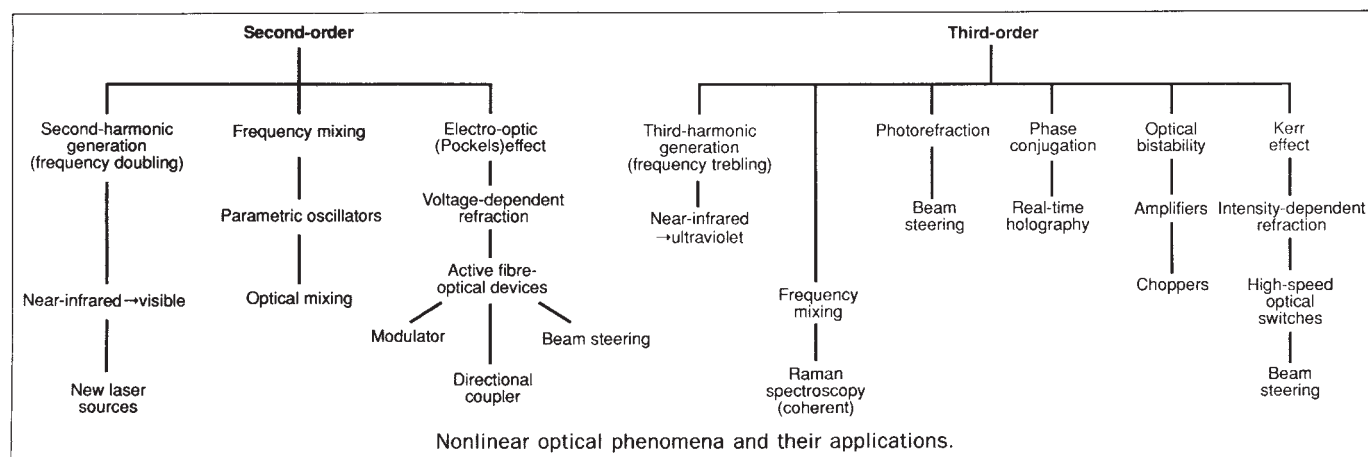
In contrast with resonant processes, with which switching times are determined by excited-state lifetimes, nonresonant processes are essentially instantaneous. Moreover, nonresonant processes occur without light attenuation or heating effects, and the magnitude of such effects does not vary dramatically with wavelength, so long as this is far

from any resonance transitions.

Theoretical predictions³ in the 1970s of large nonresonant ground-state optical nonlinearities in polyenes generated a lot of excitement: initially, they suggested that optical nonlinearities would increase without limit as the length of the polyene segments increased. More realistic theoretical considerations established that finite electron correlation-length effects would limit the nonlinearities. These self-localization phenomena are the product of competition between electron-electron and electron-phonon interactions. As shown by Garito and co-workers⁴, ground-state optical nonlinearities are also limited by the cancellation of contributions of opposite signs. The unfortunate result of these practical limitations is that nonresonant third-order optical nonlinearities have, to date, been insufficient for device development.

Fortunately, as has been shown by Garito and co-workers², this cancellation of contributions does not occur for some excited states, and excited-state optical nonlinearities are predicted to be several orders of magnitude greater than ground-state values for polyenes. This is what they verify experimentally in this issue¹. Although the probe beams in this arrangement are far from resonance, so that they are not absorbed and do not heat the lattice, heating can arise from the pump-beam absorption that produces the excited state populations. This is something which must be considered in working towards devices. The lifetime of the excited state is another feature that must also be taken into account in practice. Heating and lifetime limitations are obviously undesirable, but they may be an acceptable price for the desperately needed gains in magnitude of optical nonlinearity.

Even with the enhancements reported by Garito and co-workers, optical nonlinearities for polyenes are not sufficient for general device applications. Rather than providing a material immediately ready for application to the development



of nonlinear optical devices, the work of Garito and co-workers opens a new avenue of research into the development of materials which may ultimately be suitable. Other approaches include cascading effects, in which third-order optical nonlinearity is strengthened via a cascaded second-order optical

nonlinearity⁵, and morphological resonances such as might be expected for chromophore-coated latex particles. □

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1. Rodenberger, D. C., Heflin, J. R. & Garito, A. F. *Nature* **359**, 309–311 (1992).
2. Zhou, Q. L., Heflin, J. R., Wong, K. Y., Zamani-Khamiri, O. & Garito, A. F. in *Organic Molecules for Nonlinear Optics and Photonics* (eds Messier, J., Kajzar, F. & Prasad, P.) 239–262 (Kluwer, Dordrecht, 1991).

3. Cojan, C., Agrawal, G. P. & Flytzanis, C. *Phys. Rev. B* **15**, 909–925 (1977).
4. Heflin, J. R., Cai, Y. M. & Garito, A. F. *Opt. Soc. Am. B* **8**, 2132–2147 (1991).
5. Prasad, P. N. & Williams, D. J. *Introduction to Nonlinear Optical Effects in Molecules and Polymers*, 196 (Wiley-Interscience, New York, 1991).

RETINOBLASTOMA

For our eyes only

Ed Harlow

THREE papers in this issue^{1–3} (pages 288, 295 and 328) tease, test and twist our imagination. All three describe the biological consequences of losing a functional retinoblastoma gene (*Rb-1*) in the mouse. With some minor variations, all three groups report that the results are dire — death *in utero* at about embryonic day 12 to 13. The cause of death seems to lie in the inability of certain cell lineages, particularly in the central nervous system and erythrocytes, to differentiate correctly. So, for what seems like the first time in a while, a gene knock-out in the mouse has given us what on the surface appears to be the expected answer. The loss of a known tumour-suppressor gene should have nasty consequences. And the retinoblastoma gene knockouts are devastating.

Mutations

Interest in the retinoblastoma gene has remained high for two decades. In 1971 Knudson suggested that human retinoblastomas, childhood tumours of the developing retina, might be generated by as few as two rate-limiting mutations⁴. Comings proposed that the two mutations might occur in the alleles of the same gene and thus would lead to the loss of the gene product⁵. Support for these hypotheses came with the cloning of the *RB-1* gene from humans^{6–8}, the demonstration that retinoblastomas characteristically contain mutations in both alleles of *RB-1*^{6–8}, and the re-establishment of some aspects of growth control by introducing wild-type *RB-1* into *RB*-mutant cells⁹.

The molecular definition of the primary lesions in retinoblastomas sets the standard for the tumour-suppressor genes. The main characteristic of these genes is that mutations inactivate protein function and thereby promote tumorigenesis. This suggests that the products of tumour-suppressor genes normally act

as negative regulators of proliferation. This group of genes now includes *p53*, *WT-1* (Wilm's tumour), *DCC* (deleted in colon carcinoma), *NF-1* (neurofibrosarcoma) and *APC* (adenomatous polyposis coli).

Other observations also have suggested that the retinoblastoma protein (*Rb*) plays a central role in controlling cell proliferation. As part of their ability to stimulate cell division, DNA tumour viruses synthesize proteins that bind to and inactivate the protein, thus functionally mimicking the loss of *RB-1* seen in human tumours^{10–12}. More recently, we have begun to understand portions of the signal transduction pathways in which *Rb* acts. Through poorly known mechanisms, which probably include phosphorylation by cell cycle-regulating kinases^{13–18}, *Rb* is able to modulate the action of transcription factors such as *E2F*^{19–22}. Interaction of *Rb* with *E2F* apparently blocks transactivation of certain growth-regulating genes, while loss of *Rb* allows these genes to be transcribed at inappropriate times. As well as learning the details of *Rb* action, the major questions on the biochemistry of *Rb* include understanding the upstream signals that control its function and determining which genes are regulated by *E2F* or *E2F*-like factors.

The effects of germline deletions of the *Rb-1* gene are a dramatic test of *Rb*'s action in normal development. Although not all tumour-suppressor genes have been tested in this manner, the results with *Rb-1* are quite different from those with *p53* (ref. 23). Many would have predicted that the loss of *p53* protein would have eliminated an essential function in controlling cell division or differentiation. Surprisingly, mice that have no *p53* appear to develop normally. The findings with the *Rb-1*-deficient mice are far more reassuring for our concepts of tumour-suppressor genes.

But the new results^{1–3} also spring several surprises. First, death actually occurs surprisingly late in embryonic development. By embryonic day 12 in mouse development, many important lineage decisions have been made, and many millions of cell divisions have already occurred. The suggestion that *Rb* has an essential role in all normal cell divisions must now be abandoned — it just isn't that important. Perhaps early divisions are protected by a backup mechanism or maybe *Rb* just isn't needed then. But, for whatever reason, the protein is not the centre of every cell's G1-to-S transition in the cell cycle. Development is clearly altered, however, and we must shift our thinking towards *Rb*'s function in triggering cell differentiation.

Abnormalities

The types of abnormalities that occur in the *Rb*-deficient mice raise new issues as well. There seems to be no obvious reason why differentiation in the central nervous system and erythrocytes should suffer uniquely from loss of *Rb*. These cells come from completely different embryonic lineages, are affected in totally different regions in the embryo and serve different functions for the animal. The only connection between them seems to be that *Rb*'s role is rate limiting. However, this might provide a key. It seems unlikely that *Rb* performs different biochemical functions in these cells, and if its action is indeed similar then perhaps the types of decisions that the cells face are similar. An obvious link is that both neurons and erythrocytes cease division and are terminally differentiated quite early in development. Here, the possibility of a role for *Rb* protein in arresting cell proliferation is appealing, particularly if differentiations in the central nervous system and erythrocytes present the first decisions of this type or of this importance.

Another surprise in this week's papers is the absence of retinal tumours in mice carrying one mutant *Rb-1* allele. In humans the chance of mutating the two *RB-1* alleles during somatic development of the retina is low (about 1 tumour in 20,000 births). However, if one mutant *RB-1* allele is inherited from a parent, the chance of developing a retinoblastoma becomes very high, reaching about 85 per cent (with an average of three tumours per birth). Yet none of the heterozygous mice carrying a mutant allele of *Rb-1* develop retinoblastomas.

In the past, pesky colleagues have frequently asked why mice never develop retinoblastomas. Even considering the low frequency in humans of generating both somatic mutations in one cell, it was surprising that these tumours aren't found in mice or in other vertebrates.

You can quickly estimate the number of divisions needed to develop a human retina and multiply this by the frequency of the disease in humans. Then compare this with the number of divisions needed for a mouse retina and an estimate of the total number of mouse births. The numbers suggest that some mouseketeer or animal breeder should have seen a retinoblastoma. But naturally occurring retinoblastomas appear to be confined to humans. Why us?

The biochemical reasons are obscure, but the human retina seems to depend heavily on the accurate function of Rb. Apparently, other vertebrate retinas do not. This is not to say that the other vertebrates don't use Rb. The homozygous deletions in mice say emphatically that they do. From studies of the heterozygous *Rb-1* mice it seems that Rb protein is particularly important in certain adult mouse tissues as well. Jacks *et al.*² show that these heterozygotes develop pituitary tumours. So predisposition in mice is for pituitary tumours, in humans for retinoblastomas.

One of the quirks of science is how much timing matters in the development of ideas. If the retinoblastoma gene had been identified in mice first, it would be known as pituitarioma or CNSless or some other trick of our nomenclaturists. But whatever the course that has been followed, we have been led to an intriguing gene and its product. There are more surprises in store, I'm sure — don't you just love experiments that raise more questions than they answer? □

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1. Lee, E. Y.-H. *et al.* *Nature* **359**, 288–294 (1992).
2. Jacks, T. *et al.* *Nature* **359**, 295–300 (1992).
3. Clarke, A. R. *et al.* *Nature* **359**, 328–330 (1992).
4. Knudson, A. *Proc. natn. Acad. Sci. U.S.A.* **68**, 820–823 (1971).
5. Comings, D. *Proc. natn. Acad. Sci. U.S.A.* **70**, 3324–3328 (1973).
6. Friend, S. H. *et al.* *Nature* **323**, 643–646 (1986).
7. Lee, W. H. *et al.* *Science* **235**, 1394–1399 (1987).
8. Fung, Y. K. *et al.* *Science* **236**, 1657–1661 (1987).
9. Huang, H. J. *et al.* *Science* **242**, 1563–1566 (1988).
10. Whyte, P. *et al.* *Nature* **334**, 124–129 (1988).
11. DeCaprio, J. A. *et al.* *Cell* **54**, 275–283 (1988).
12. Dyson, N., Howley, P. M., Munger, K. & Harlow, E. *Science* **243**, 934–937 (1989).
13. Buchkovich, K., Duffy, L. A. & Harlow, E. *Cell* **58**, 1097–1105 (1989).
14. Chen, P.-L., Scully, P., Shew, J.-Y., Wang, J. & Lee, W.-H. *Cell* **58**, 1193–1198 (1989).
15. DeCaprio, J. A. *et al.* *Cell* **58**, 1085–1095 (1989).
16. Mihara, K. *et al.* *Science* **246**, 1300–1303 (1989).
17. Lin, B. T., Gruenwald, S., Morla, A. O., Lee, W. H. & Wang, J. Y. *EMBO J.* **10**, 857–864 (1991).
18. Lees, J. A., Buchkovich, K. J., Marshak, D. R., Anderson, C. W. & Harlow, E. *EMBO J.* **10**, 4279–4290 (1991).
19. Bandara, L. R., Adamczewski, J. P., Hunt, T. & La, T. N. *Nature* **352**, 249–251 (1991).
20. Bagchi, S., Weinmann, R. & Raychaudhuri, P. *Cell* **65**, 1063–1072 (1991).
21. Chellappan, S., Hiebert, S., Mudryj, M., Horowitz, J. & Nevins, J. *Cell* **65**, 1053–1061 (1991).
22. Chittenden, T., Livingston, D. & Kaelin, W. *Cell* **65**, 1073–1082 (1991).
23. Donehower, L. *et al.* *Nature* **356**, 215–221 (1992).

Breaking out in spots

Harold Zirin

HIGH-RESOLUTION images of the Sun's surface, presented by C. U. Keller in this issue (*Nature* **359**, 307–308; 1992), show that it is covered by magnetic spots, diminutive relations of sunspots.

As discovered by Hale at the beginning of this century, sunspots are associated with strong magnetic fields, up to 3,500 gauss. But other apparently weaker fields cover the solar surface. Since 1973, J. O. Stenflo (*Sol. Phys.* **32**, 41–63; 1973) has championed the idea that

would be destroyed if they were weaker. In the original model, the pressure of the magnetic fields was contained because they were localized in 'invisible sunspots'. These, indeed, were eventually made visible at the Big Bear Observatory (H. Zirin & H. Wang *Astrophys. J.* **385**, L27–L29; 1992).

Also, fields never appear uniform in high-resolution magnetograms: rather elements as small as the limiting resolution can be seen. Keller's work now



John Bova/Science Photo Library

Rough with the smooth: the apparently featureless surface between sunspots is in fact crowded with tiny flux tubes no more than 180 km across.

these are in fact the poorly resolved images of much smaller and much stronger field elements. Over the years, Stenflo and his students have accumulated a lot of evidence supporting this case, until only a few die-hard conservatives, myself included, harbour doubts.

The problem is one of spatial resolution: our atmosphere, heated by the Sun, is so turbulent that it is hard to see anything smaller than 1 arcsecond, or 700 km, across. Thus the small elements of strong field cannot be seen as such and indirect techniques must be used. In his original work Stenflo used the ratio of magnetic fields measured in two atomic spectral lines which respond differently (through the Zeeman effect) to external fields and showed that the results could be explained only if the fields were very strong.

For the present work, Keller has used super-high-resolution images obtained from the Canary Islands by speckle-interferometric techniques to find very small bright points associated with magnetic fields. The smallest point resolved is remarkable, only 180 km across. The magnetograms show similarly high resolution.

The logic of the strong-field model is that the motions of the Sun's photosphere are so strong that small elements

shows that the elements must be less than 180 km across. The strong-field model requires field elements smaller than 100 km, the mean free path of radiation at the surface.

There are still plenty of problems. Keller finds that some magnetic elements change rapidly, something we almost never see in our data, which is more sensitive, but has lower resolution; and he finds the bright point intensity doesn't always match the magnetic field. He finds that magnetic elements over 300 km are mostly dark, which partly agrees with our results.

And although strong fields will be blurred in measurement, individual dark elements cannot absorb more than 100 per cent of the light, so the invisible spots must have a filling factor of at least 50 per cent. This requires a lower field strength. And it is remarkable that these field elements can last so long, certainly weeks, perhaps years, without breaking up. While the problem of just how big the elements are was to be solved by the Orbiting Solar Laboratory, cancelled last year, Keller's brilliant work shows that there are other means of tackling it. □

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Barbara McClintock (1902–1992)

BARBARA MCCLINTOCK, pioneering geneticist and Nobel laureate, died on 2 September in Huntington, New York, after a brief illness. McClintock recently had been fêted on her ninetieth birthday by a gathering of friends at Cold Spring Harbor and the preparation of a unique volume in her honour, *The Dynamic Genome: Barbara McClintock's Ideas in the Century of Genetics* (reviewed in *Nature* of 20 August).

McClintock was born in Hartford,

ingenious analysis of triploids. In 1931, together with Harriet Creighton, she published a landmark experiment showing that genetic crossing-over was associated with the exchange of parts of homologous chromosomes.

These discoveries established her at a young age as a talented theoretician and virtuoso experimentalist, able to perform cytological studies on organisms that others found intractable — her beautiful photographs of chromosomes

work in the 1950s and 1960s is best exemplified by a comment attributed by Mel Green to the renowned geneticist A. H. Sturtevant. Reporting to a group of interested colleagues at the California Institute of Technology about her talk at the 1951 Cold Spring Harbor symposium, Sturtevant said, "I didn't understand one word she said, but if she says it is so, it must be so!"

Her papers on transposition in maize also contained prescient observations on hitherto unknown epigenetic phenomena, presaging areas of intense current interest such as gene imprinting. She thus leaves fertile ground for a whole new generation of geneticists.

McClintock's ideas about a dynamic genome did not become generally understood until the late 1970s, when transposable elements had been discovered in many organisms and molecular techniques were available for isolation and identification of the DNA segments comprising transposons. In 1983, she received the Nobel prize for her discovery of "mobile genetic elements".

By many accounts McClintock was a very private person. All of her experiments on transposable elements in the 1940s and 1950s were carried out alone at Cold Spring Harbor, where she lived in monk-like austerity. Yet if she was private, she was also very sociable. She was a spirited conversationalist, taking every opportunity for a discussion with younger scientists, who made regular pilgrimages to spend a day with her. Many scientists fondly recall long conversations with her about philosophy, politics and art, as well as science. Children who grew up at Cold Spring Harbor Laboratory remember her as an occasional companion who accompanied them home from the school bus stop, discussing what insects did in the winter or the anatomy of walnuts.

At her ninetieth birthday party, McClintock reflected that it had become more difficult to get to know the summer visitors to Cold Spring Harbor because they whisked in for meetings and then, after a few days, drove away. She relished the days when people stayed for the whole summer, so that she could renew acquaintances or make new ones. For many scientists McClintock was a hero, someone who by her own example helped them find strength in themselves. Her burning curiosity, enthusiasm and uncompromising honesty serve as a constant reminder of what drew us all to science in the first place.

Gerald R. Fink

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Marcus Rhoades

Barbara McClintock in 1929, when she was a member of R. A. Emerson's maize genetics group at Cornell. The others (standing, right to left) are Charles Burnham, Marcus Rhoades and Emerson, with George Beadle squatting.

Connecticut, and raised in Brooklyn, New York. She received her BS (1923) and PhD (1927) degrees from Cornell University, where she continued to work as a research associate in R. A. Emerson's laboratory together with George Beadle, Marcus Rhoades and Charles Burnham — the Cornell corn group. Following a brief stint as an assistant professor at the University of Missouri, she moved to Cold Spring Harbor in December 1941. For more than 50 years, she lived and worked at the Cold Spring Harbor Laboratory under the auspices of the Department of Genetics of the Carnegie Institution of Washington.

McClintock rose quickly to prominence through a series of remarkable experiments on her favourite organism, corn (otherwise maize). In her thesis work she assigned linkage groups established by genetic analysis to cytologically identifiable chromosomes using an

remain as a testimony to her gifts as an artisan as well as a scientist. These achievements were recognized by her election to the National Academy of Sciences at the age of 42 and, a year later, her election as president of the Genetics Society of America.

McClintock's work during the 1940s firmly established her as a major figure in the history of genetics. It was then that she initiated studies leading to her discovery of transposable elements in corn. The first evidence for these unstable genetic elements came from unexpected results in her continuing research on the fate of broken chromosomes. Her discovery of movable genetic elements, though documented in the same rigorous style as her earlier work, was puzzling to her contemporaries. Yet she was held in such high esteem that her findings were accepted and even codified in genetics textbooks. The prevailing opinion about McClintock's

Rac and Rho in tune

Julian Downward

THE actin cytoskeleton is a key player in many cellular processes, including cell division and growth control. It has long been known that cytoskeletal architecture is influenced by growth factors and cellular transformation, and evidence has begun to emerge that Rho proteins, small guanine-nucleotide-binding proteins related to Ras, are involved in regulating its construction¹⁻³. In a remarkable leap forward, reported in two papers in *Cell*^{4,5}, Anne Ridley, Alan Hall and their colleagues have revolutionized our understanding of the connections between serum growth factors, GTP-binding proteins and the control of cytoskeletal structure. The inside of the black box coupling extracellular factors to actin organization has been illuminated to reveal a cascade of Ras superfamily GTPases.

Rho family

The Rho family of GTP-binding proteins is one of several groups of proteins related to the products of the *ras* oncogenes⁶. In mammals they comprise four closely related Rho proteins, two Rac proteins, TC10 and two CDC42Hs proteins, each being at least 50 per cent similar in sequence to other members of the family, but only about 30 per cent related to Ras. Like Ras, they bind GTP and catalyse its hydrolysis to GDP, presumably being active in the GTP-bound state and inactive when GDP-bound; this reaction is also stimulated by GTPase-activating proteins (GAPs) such as Rho-GAP for Rho, and Rho-GAP, Bcr and n-chimaerin for Rac⁷. In addition, Settleman *et al.* have shown that p190, a protein that associates with Ras-GAP, has GAP activity towards Rho, Rac and CDC42 proteins⁸. These GAPs are negative regulators of the Rho family proteins, but could perhaps also be downstream effectors, passing a signal on to targets within the cell.

In addition, a guanine nucleotide exchange-promoting factor, Dbl, has been identified for the Rho-like protein CDC42Hs⁹: this would be expected to activate CDC42Hs by promoting the replacement of GDP by fresh GTP from the cytosol. Other exchange factors with broader specificity have also been characterized (Rho-GDS, smg-GDS). The Rac1 protein has previously been shown to form part of a regulatory complex for NADPH oxidase in phagocytes¹⁰; the relationship of this specialized role to the more general functions described below is not clear.

Starting from their earlier observations³ that microinjection of

mutationally activated Rho protein into quiescent fibroblasts led to the rapid formation of actin stress fibres, Ridley and Hall⁴ have now shown that addition of serum results in a similar effect. They have identified the principal component of serum responsible as the mitogenic lipid lysophosphatidic acid; the neuropeptide bombesin is also active, though less potent. Formation of stress fibres in response to serum, lysophosphatidic acid or bombesin is blocked by microinjection of exoenzyme C3 transferase from *Clostridium botulinum*, which ADP-ribosylates Rho on asparagine at position 41, thereby inhibiting its function. Thus Rho mediates the control of actin stress fibre formation by extracellular factors: this pathway is likely to involve the cycling of Rho between GTP- and GDP-bound states in response to incoming stimuli.

In fibroblasts the actin cytoskeleton is made up of three distinct elements: the cortical actin network, lying beneath the plasma membrane; actin stress fibres, which emerge from focal adhesions where cell-surface integrins bind to the extracellular matrix; and cell-surface projections, such as membrane ruffles.

Ridley *et al.*⁵ also report that a number of growth factors, such as epidermal growth factor, platelet-derived growth factor, insulin and thrombin induce formation of stress fibres much more slowly than serum but also induce rapid membrane ruffling. Lysophosphatidic acid does not cause membrane ruffling, but it is seen when activated Rac protein is microinjected into confluent fibroblasts, while a dominant negative mutant of Rac (mutated from serine to asparagine at residue 17) blocks growth-factor-induced membrane ruffling and stress fibre formation.

Rac activation

These growth factors therefore seem to activate Rac, leading to rapid induction of membrane ruffles, activated Rac in turn causing activation of Rho and stress fibre formation (see figure). This hierarchy of GTP-binding proteins is extended by the observation that activated Ras causes both membrane ruffling and stress fibre formation, although microinjection of neutralizing antibodies shows that Ras is not obligatory for the growth factor effects on the cytoskeleton.

These studies identify Rho and Rac as central regulatory molecules that control the structure of two forms of actin microfilaments within the cell in response to extracellular factors. The

IMAGE
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REASONS

A model for Ras superfamily control of the actin cytoskeleton. The growth stimuli listed at the right cause accumulation of Rho or Rac in the active GTP-bound state, either by stimulation of guanine-nucleotide-exchange factors (GDS or Dbl-like proteins) or by inhibition of specific GTPase-activating proteins (Rho-GAP, Bcr, n-chimaerin and related proteins). The activated GTP-binding proteins then cause membrane ruffling (Rac) or stress fibre formation (Rho), possibly acting through one of the GTPase-activating proteins. PDGF, platelet-derived growth factor; EGF, epidermal growth factor; LPA, lysophosphatidic acid.

mechanisms involved have yet to be resolved in detail, but there are a number of intriguing possibilities. Upstream regulation of Rho or Rac could occur through regulation of GAPs: Rho-GAP is inhibited by lysophosphatidic acid, although not as strongly as by phosphatidic acid, which is far less potent in causing cytoskeletal changes, while n-chimaerin (a Rac-GAP) binds phorbol esters and diacylglycerol which are also activators of membrane ruffling. Regulation could also be effected through putative exchange factors related to Dbl such as Vav, which contains SH2 and SH3 domains, Bcr, a potential bifunctional molecule also possessing Rac-GAP activity, and Ras-GRF, which is in addition an exchange factor for Ras¹¹.

The downstream effects of Rac and Rho on the actin cytoskeleton might also be mediated by their GAPs. It is intriguing that many proteins with proven or suspected Rho family GAP activity either possess Src-homologous SH2 and SH3 domains (phosphatidylinositol 3-OH kinase p85 subunit¹²) or bind to proteins that possess these domains (p190 to Ras-GAP¹³, Bcr and 3BP-1 to c-Abl^{14,15}). SH2 domains bind to phosphotyrosine-containing proteins; the role of SH3 is less clear, although it is frequently found in actin-binding proteins leading to the conjecture that it mediates attachment to the cytoskeleton¹⁶. Might SH3 domains provide the points at which signalling pathways from Rho and Rac lead into control of the actin cytoskeleton?

The explosion in discovery of proteins with SH2, SH3, Rho-GAP and Dbl sequence motifs suggests that many of the remaining pieces of the jigsaw puzzle of how Rho and Rac control the cytoskeleton have already been identified. The challenge now is to slot them into place to complete this elegant picture. □

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Merely the tip of the ice core

David Peel

EARLIER this summer, a team of over 30 scientists from eight European nations, collaborating in the Greenland Ice Core Project (GRIP), successfully drilled an ice core through the deepest part of the Greenland ice sheet. After four summers of intense effort at a temporary camp established on the summit of the ice sheet, silty ice and finally bedrock was reached on 12 July at a depth of 3028.6 m, where the estimated age of the ice is at least 200,000 years. The record ex-

years. In particular, they find that sudden temporary warmings which, from previous data², appeared to characterize the last 30,000 years of the last ice age really did occur, raising mean temperatures by up to 7 °C for up to 2,000 years.

Previous evidence of these variations, obtained from other Greenland cores, may have been distorted because the drilling sites were not ideal scientifically, being constrained by logistical considerations: their ice from the last glaciation now lies close to the influence of bedrock. For the first time, drilling at Summit was carried out at a scientifically optimum site where the critical glacial-interglacial transition lies at only half the depth of the ice sheet. Indeed, even seasonal oscillations in several chemical parameters can be detected at and below the transition, promising that the records from this core will be dated accurately even well into the last glaciation. (The provisional timescale presented by Johnsen *et al.* is likely to be tightened as new data become available, but it is interesting that the Younger Dryas, the last oscillation, seems to have ended significantly earlier than previously thought. Improvements in dating will be vital in establishing the synchronicity between records of this event in different media, such as marine and terrestrial sediments, and from different regions — pointers to underlying causes.)

The new stable-isotope data show convincingly that the previously reported abrupt changes are indeed a direct reflection of climatic flickers and are not an artefact of ice dynamics. Available evidence suggests that the isotope shifts represent actual temperature changes. On each occasion, the inferred warming, by around 7 °C, seems to have been very rapid, taking just a few decades, but the return to fully glacial conditions was comparatively slow; similar timescales have been seen with the Younger Dryas³. The timing of these events is apparently random, leading the authors to speculate that they may reflect an important element of chaos in the ocean/atmosphere system acting on long timescales. Clearly, the data suggest that the dynamics of the climate system may depend rather crucially on the state of the climate system at a particular time and caution will be needed when trying to draw lessons for any future climate warming.

Nevertheless, although the climatic context at the time of these events differed greatly from current conditions, if they can be characterized faithfully they should provide important case studies for testing the performance of climate



Inside the GRIP hut at Summit. The 11-m Danish drill 'ISTUK' is in its raised position. On each run, it cuts 2.5-m of core.

tends through the last interglacial period and into the last but one ice age — probably the longest ice-core record possible in the Northern Hemisphere.

The main aim of GRIP is to reconstruct the history of climate and environmental change through the last glacial cycle in unprecedented detail, as a basis for understanding some of the mechanisms involved in past climate changes. On page 311 of this issue¹, Johnsen *et al.* lay the groundwork for an anticipated stream of publications based on the analysis of more than 30 constituents contained in the ice. Drawing on data from the core which had been drilled down to a depth of 2,321m (about 40,000 years ago) by the end of the 1991 field season, they give a taste of the exciting information that can be expected from the complete core over the next few

Nars Silis

1. Rubin, E. J., Gill, D. M., Boquet, P. & Popoff, M. R. *Molec. cell. Biol.* **8**, 418–426 (1988).
2. Chardin, P. *et al.* *EMBO J.* **8**, 1087–1092 (1989).
3. Paterson, H. F. *et al.* *J. Cell Biol.* **111**, 1001–1007 (1990).
4. Ridley, A. & Hall, A. *Cell* **70**, 389–399 (1992).
5. Ridley, A., Paterson, H. F., Johnston, C. L., Diekmann, D. & Hall, A. *Cell* **70**, 401–410 (1992).
6. Downward, J. *Trends biochem. Sci.* **15**, 469–472 (1990).
7. Diekmann, D. *et al.* *Nature* **351**, 400–402 (1991).
8. Settleman, J., Albright, C. F., Foster, L. C. & Weinberg, R. A. *Nature* **359**, 153–154 (1992).
9. Hart, M. J., Eva, A., Evans, T., Aaronson, S. A. & Cerione, R. A. *Nature* **354**, 311–314 (1991).
10. Abo, A. *et al.* *Nature* **353**, 668–670 (1991).
11. Downward, J. *Nature* **358**, 282–283 (1992).
12. Fry, M. J. *Curr. Biol.* **2**, 78–80 (1992).
13. Settleman, J., Narasimhan, V., Foster, L. C. & Weinberg, R. A. *Cell* **69**, 539–549 (1992).
14. Pendergast, A. M., Muller, A. J., Havlik, M. H., Maru, Y. & Witte, O. *Cell* **66**, 161–171 (1991).
15. Cicchetti, P., Mayer, B. J., Thiel, G. & Baltimore, D. *Science* **257**, 803–806 (1992).
16. Musacchio, A., Gibson, T., Lehto, V. P. & Saraste, M. *FEBS Lett.* **307**, 55–61 (1992).

RÉSUMÉ

models during periods of rapid climatic change. Most important is the prospect that this core will give the best opportunity so far to determine whether simultaneous changes in the greenhouse gases were involved — first to confirm the somewhat incomplete evidence from the earlier cores that important changes in greenhouse gas composition occurred during these events⁴; and second, taking advantage of the much greater resolution of this core, to determine whether there were significant leads or lags with respect to the temperature changes.

A feature of the GRIP project was the amount of analytical technology taken into the field, allowing a broad spectrum of constituents to be analysed alongside the drilling operation, often at high resolution (in some cases at millimetre-resolution) along the length of the core. Thus continuous profiles including d.c. and a.c. electrical properties (relating to hydrogen ion and total neutral salt contents) and microparticle, ammonium, nitrate and hydrogen peroxide contents are already available and have contributed to the multi-parameter dating strategy. Many of these species are relevant to atmospheric chemistry and should help to elucidate the link between climate and other environmental changes.

One of the first surprises was the discovery of sporadic high-concentration

spikes of ammonium together with several organic acids, especially formate. Legrand and others⁵ have hypothesized that these events are fingerprints of biomass burning events in the northern high latitudes, which may have been regulated by climatic conditions in the past. Further surprises are likely, and their relation to climate must be studied in the historical perspective, where unlikely events become possible simply because of the passage of time.

Eagerly awaited will be the first data through the last interglacial, when conditions around 126,000 years ago were up to 2 °C warmer than now. This should give us the first detail from the Northern Hemisphere on climate and associated environmental responses during the last period of the Earth's history when conditions were analogous to those predicted for the near future. □

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1. Johnsen, S. J. *et al.* *Nature* **359**, 311–313 (1992).
2. Oeschger, H. & Arquit, A. in *Global Changes in the Past* (ed. Bradley, R. S.) 175–200 (UCAR/Office for Interdisciplinary Earth Studies, Boulder, Colorado, 1991).
3. Dansgaard, W. *et al.* *Nature* **339**, 532–533 (1989).
4. Stauffer, T., Stauffer, B. & Oeschger, H. *Ann. Glaciol.* **10**, 167–170 (1988).
5. Legrand, M., De Angelis, M., Stauffer, T., Neftel, A. & Stauffer, B. *Geophys. Res. Lett.* **19**, 473–475 (1992).

OZONE DEPLETION

Volcanic aerosols implicated

Guy Brasseur

Is stratospheric ozone vulnerable to volcanic eruptions? Yes, according to reports detailing depletion recorded last year. In *Geophysical Research Letters*¹, Grant *et al.* describe *in situ* balloon measurements and satellite measurements showing that ozone levels were low in the mid-latitudes in the months following the eruption of Mount Pinatubo, Philippines. And on page 283 of this issue², Hofmann *et al.* attribute unexpected changes in the ozone hole that appeared over Antarctica last year to aerosols transported from the less well known eruption of Mount Hudson, in Chile.

The effect of volcanoes on ozone has been widely debated^{3,4} since evidence was produced⁵ that ozone abundance decreased following the eruption in 1982 of El Chichón in Mexico. The argument presented in the early 1980s was that volcanoes release chlorine into the atmosphere (mainly in the form of hydrogen chloride) and that chlorine has the potential to destroy ozone efficiently in the stratosphere. This natural source,

however, affects the stratosphere much less than the anthropogenic source provided by industrially manufactured chlorofluorocarbons (CFCs). In fact, hydrogen chloride is rapidly removed from the troposphere by washout processes in clouds, whereas the CFCs are not. Thus, CFCs easily penetrate into the stratosphere where they are photolysed by solar ultraviolet radiation and release reactive chlorine atoms.

The gas-phase reactions involved in the destruction of stratospheric ozone by chlorine account only for a small fraction of the ozone depletion recorded since 1980. In addition, the largest ozone trends (approximately 10–30 per cent per decade), which have been observed between the tropopause and 20 km altitude, are not reproduced by models based on gas-phase chemistry alone. The discrepancy is now commonly attributed to the role of solid particles (such as ice crystals in polar stratospheric clouds) or small liquid droplets (such as the sulphate aerosol particles) observed in the lower stratosphere. As a result of

Beyond the fringe

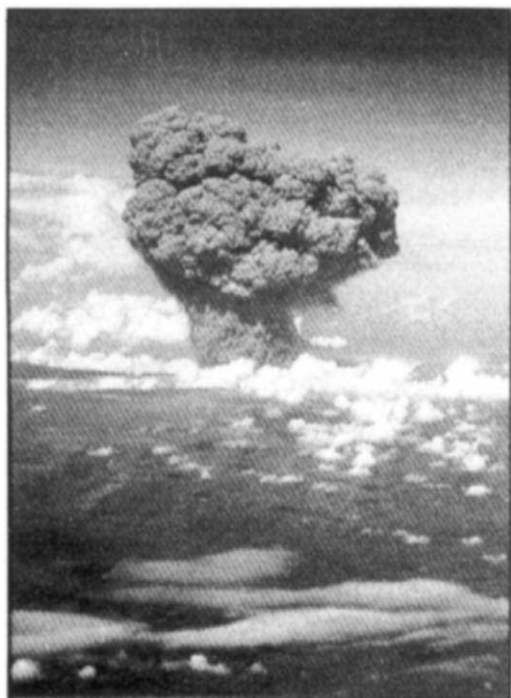
BEYOND Pluto and Neptune (themselves 4.5 billion kilometres, or 30 astronomical units, away), D. Jewitt and J. Luu believe they have discovered the most distant member of the Solar System yet (*IAU Circ.* No. 5611; 1992). Glorifying in the name of 1992 QB1, the object was first spotted from the 2.2-metre telescope at Mauna Kea on 30 August, and was tracked for the next two days. It is so faint that it will now not be seen until the next moonless night. Assuming that its albedo is that typical for comets (4 per cent) the authors reckon from its brightness that 1992 QB1 is a mere 200 km across. They note that it is remarkably red, suggesting that its icy surface is rich in organic materials. The exact orbit will not be clear for several months, but 1992 QB1 seems to be 37–59 astronomical units away. One possibility is that the object is the first-identified occupant of the Kuiper Belt, a much discussed but purely hypothetical reservoir of comets at the edge of the visible Solar System.

Eating in

AN unlikely breed of miners is described in the *Mineralogical Society Bulletin* (No. 96, 3–4; 1992) by R. J. Bowell. It seems that elephants are wont to traverse a perilous ledge, worn down by generations, 345 metres up the side of a cliff on Mount Elgon, on the Kenya–Uganda border, to reach a natural cave known as Kitum. Their literal quarry is deposits of zeolites (alumina silicate minerals) precipitated in the mountain's volcanic basalts by hydrothermal activity. These they excavate with their trunks, passing the lumps around the herd to be eaten for the sodium and calcium salts they contain. Like all gourmets, the elephants are particular about their zeolites; the choicest morsels are those that have been seasoned by reaction with bats' guano.

Cosmic undoing

TEXTURE, one of the obscure palliatives devised by cosmologists to account for the present structure of the Universe, is called into question in papers by M. Kamionkowski and J. March-Russell, and R. Holman *et al.* (*Phys. Rev. Lett.* **69**, 1485–1488, 1489–1492; 1992). Like magnetic monopoles or cosmic strings, textures are topological defects embedded in space. Unlike them, textures unwind as different regions of space come into contact; this is how they created the seeds of cosmological structure shortly after the Big Bang. But the new work shows that textures are unlikely to last long enough to do the job. To be cosmologically useful, texture is so delicately balanced that gravitational effects, even those due to primordial Planck-mass (20 μ g) black holes, will prevent it from forming.



Popperfoto/Reuter

The plume from Mount Pinatubo, seen here on 12 June 1991, rose over 10 km; volcanic aerosols reached well into the stratosphere.

heterogeneous reactions occurring on the surface of these particles, nitrogen oxides are transformed into nitric acid and, especially in the coldest regions of the lower stratosphere, the unreactive and most abundant forms of chlorine molecules (for example, ClONO_2) are partly converted into reactive radicals. Thus, under these circumstances, the atmosphere shifts from a situation where the ozone loss is controlled primarily by nitrogen oxides to a situation where it becomes more readily determined by the abundance of chlorine.

During non-volcanic periods, the aerosol load in the atmosphere remains relatively limited but after major volcanic eruptions, such as those of El Chichón in April 1982 and Mount Pinatubo in June 1991, it can be raised by one or two orders of magnitude⁶, so that heterogeneous reactions occur at a much faster rate. Thus, as shown by models based on our current knowledge of the chemical kinetics involved, the ozone molecules of the lower stratosphere should become more vulnerable to atmospheric chlorine and so to man-made CFCs. The analysis by Grant *et al.*¹ of ozone profiles measured at Brazzaville, Congo (4° S), Ascension Island (8° S), and Natal, Brazil (6° N) after Pinatubo suggests that the ozone concentration in the 3–6 months following the eruption was reduced by as much as 15–20 per cent in the altitude range (approximately 24–25 km) where the largest volcanic aerosol loading was observed.

These significant reductions in ozone could potentially result from hetero-

geneous conversion of NO_x on the surface of sulphate aerosols. Alternatively, heating in the aerosol layer may have drawn ozone-poor, tropospheric air into the lower stratosphere⁷ so that the change was associated with transport. Or perturbations of solar radiation fields by the aerosol layer⁸ may have reduced the rate of photochemical production of ozone.

Although heterogeneous chemical mechanisms on sulphate aerosols are expected to be most efficient near the polar vortex, no convincing evidence of significant ozone depletion, at these high latitudes, associated with the eruption of Mount Pinatubo has yet been produced. The paper in this issue by Hofmann *et al.*² reports for the first time unusually high ozone reduction in Antarctica during September 1991 at altitudes where polar stratospheric clouds are usually not observed. Ozone reduction approaching 50 per cent was detected both in the 11–13 km and

25–30 km regions, resulting in an ozone column abundance 10–15 per cent lower than in previous years.

Different potential explanations are invoked by the authors, including meridional and vertical transport, and homogeneous chemical loss which is found to account for at most a 10 per cent reduction in ozone at 30 km compared with 1980. After careful analysis, Hofmann *et al.* conclude that the depletion below 12 km results from the presence of large quantities of sulphate aerosols produced from the sulphur injected during the eruption of Mount Hudson in Chile (46° S) in August 1991. The ozone depletion observed above 25 km altitude appears to be associated with transport of air from the Wedell Sea and the Palmer Peninsula area where polar stratospheric clouds seem to have been produced at relatively high altitude by mountain lee waves. □

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1. Grant, W. B. *et al. Geophys. Res. Lett.* **19**, 1109–1112 (1992).
2. Hofmann, D. J. *et al. Nature* **359**, 283–287 (1992).
3. Hofmann, D. J. & Solomon, S. *J. geophys. Res.* **94**, 5029–5041 (1989).
4. Brasseur, G. P., Granier, C. & Walters, S. *Nature* **348**, 626–628 (1990).
5. Jäger, H. & Wege, K. *J. atmos. Chem.* **10**, 273–287 (1990).
6. World Meteorological Organization. Scientific Assessment of Ozone Depletion: 1991, Global Ozone Research and Monitoring Project, Report No. 25 (1992).
7. Brasseur, G. & Granier, C. *Science* **257**, 1239–1242 (1992).
8. Michelangeli, D. V., Allen, M. & Yung, Y. L. *J. geophys. Res.* **94**, 18,429–18,443 (1989).

DAEDALUS

Sound moves

DAEDALUS is dissatisfied with existing loudspeakers. He wants to transduce electricity to sound directly by passing a modulated electric current through the air. This should heat and expand it at the sonic frequency. The problem, of course, is that air does not normally conduct electricity. One answer might be to pass sparks through it. If the sparks were passed at the 44.1 kilohertz digitization rate of a standard digital recording, each spark having the precise energy of the digital signal at that instant, the spark gap should radiate perfect high-fidelity digital sound.

To generate really loud sound in this manner would need worryingly intense sparks. It might be better to use a strong continuous electric current, such as a carbon arc or a silent electric discharge, and modulate it with an analogue sound signal. But Daedalus prefers the idea of building up a strong digital signal by many small additions of energy. This is the idea behind his new digital sonic travelling-wave tube.

It consists of a tube with a series of electrode pairs, spaced at 7.8-millimetre intervals down its length. At the instant of starting, the front electrode pair carries an electric discharge with intensity proportional to the first sample in the digital signal. The next electrode pair has a discharge reflecting the next sample in the signal, and so on down the tube. Then 22.7 microseconds later (that is, one digitization interval at 44.1 kHz), all the discharge intensities are stepped up the tube to the next electrode pair. The whole pattern moves 7.8 mm up the tube, while the last electrode pair acquires the next digital sample in the stream. Now sound travels 7.8 mm in 22.7 μs . So sound travelling up the tube keeps pace with its moving digital representation, and is reinforced at each step. The system acts as a sonic travelling-wave tube amplifier, and emits from its muzzle a beam of perfect, highly directional sound.

This elegant loudspeaker will transform sound reproduction. High-powered versions will direct targeted announcements at the platforms of railway stations and shout advice to sportsmen on the field. Smaller versions will grace our lecture halls and living rooms. On the smallest scale, little travelling-wave tubes may serve as the earpieces of deaf aids and personal tape players. As simple open tubes, they won't prevent the wearer hearing the sounds around him, and as unidirectional devices they won't radiate the wrong way out into the air. The only snag is that they have to be many multiples of 7.8 mm long. Some sort of horned 'Viking helmet' design seems indicated.

David Jones

Rabies in wild dogs

SIR — The African wild dog *Lycaon pictus*, probably the most endangered large carnivore in Africa¹, has been the subject of conservation research since 1985 in Serengeti National Park and Ngorongoro Conservation Area in Tanzania, and since 1987 in the Masai Mara region of Kenya. In 1989, a Kenyan pack which died from rabies², a disease not previously confirmed in wild dogs, included some handled by researchers for radio collaring³ and vaccination against rabies. Between 1985 and 1990 in the two conservation areas, four of eight unvaccinated study packs (a total of 58 dogs) died 2–5 months after radio collaring, with rabies confirmed in one pack in 1990 (ref. 4).

Following these losses, an attempt was made to vaccinate the remaining study packs in both countries using an inactivated vaccine delivered by air-pressurized darts. All study packs ($n=7$) died or disappeared within a year of vaccination. Although no known outbreak of rabies occurred in other wildlife, and tissue samples were not available in the conservation areas, rabies was again suspected. However, packs not vaccinated and without radio collars still existed in or near the study areas.

Serum samples taken up to 2 years before vaccination showed that packs had been exposed to rabies with some individuals carrying significant, possibly protective levels of rabies-neutralizing antibody⁴. This begs the question "why vaccinate?"

The feature common to all packs was 'handling'. Many mammalian species carry latent viruses, including rabies⁵, which can be reactivated by stress in some cases. Handling-induced stress, as measured by highly elevated peripheral serum cortisol concentrations, results from immobilization of captive wild dogs⁶. Corticosteroids tend to inhibit the body defences, which prevent latent infections from becoming apparent⁷. This stress mechanism perhaps reactivates rabies virus latent in 'handled' carrier dogs with the disease spreading within, but not between, the widely separated

packs, by oral social contact. Losses of all packs after vaccination, together with sporadic pack deaths after collaring, could be explained by this hypothesis, as any carrier dog(s) would be 'hit' during whole pack vaccination but only selected by chance for radio collaring.

It is possible that rabies virus persists in wild dogs in a normal host-parasite relationship^{5,8} with some naturally immune individuals. This stable system could be disrupted by handling-induced stress in some individuals, resulting in early death⁹ of dogs in packs, and vaccine-induced delay¹⁰ in emergence of rabies in the vaccinated packs.

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Origin of rodents and guinea-pigs

SIR — Our maximum parsimony (MP) analysis¹ of protein sequence data suggested that the order Rodentia may not be monophyletic but that the guinea-pig-like rodents (Caviomorpha) may have branched off earlier than the separation of the rat-like rodents (Myomorpha) from the primates. Our hypothesis, represented as ((Myomorpha, Primates), Caviomorpha), will be called tree III; the traditional view ((Myomorpha, Caviomorpha), Primates), tree I; and the third alternative ((Caviomorpha, Primates), Myomorpha), tree II. But in a maximum likelihood (ML) analysis of a similar set of protein sequences Hasegawa *et al.*² did not find significant preference for tree III. Arguing that the ML method withstands the effect of unequal evolutionary rates among lineages, they

concluded that our study may represent an example of the unequal rate effect on parsimony analysis. We would like to make three comments.

First, the ML method is model-dependent. For example, for α -crystallin A, the difference in ML value between trees I and II is only 0.4 for the Dayhoff model of amino-acid substitution, but 6.3 and 5.9 for the proportional and Poisson models, respectively².

Second, for the ten proteins used, the ML method supports tree I for three proteins, tree II for four proteins, and tree III for three proteins, if the Dayhoff model is used². This means that for most of the proteins the ML method fails to identify the true tree, regardless of which of the three trees is the real one. The same conclusion holds for the other two models of amino-acid substitution. This fact contradicts the claim² that the ML method is robust against the effect of unequal rates.

Third, the unequal rate effect is stronger for divergent sequences than for well conserved ones. The table shows the degree of divergence from the outgroup sequence to the human, myomorph and guinea-pig sequences for each protein. As β -globin has an evolutionary rate close to the mean rate for mammalian proteins³, it may be used as a reference. Let us therefore take α -crystallin A, α -globin, β -globin, lipoprotein lipase and lipocortin as conservative proteins (group I), because their degree of divergence from the outgroup sequence is smaller than or close to that for β -globin. For all these proteins the ML and MP methods are congruent, and both support tree III, except for α -crystallin A (ref. 2). In contrast, for the other five proteins in the table, which may be considered as nonconservative (group II), the ML and MP methods often do not support tree III; when all the proteins in group II are considered together, the ML method supports tree I, whereas the MP method supports tree II. Thus, if tree I is indeed the true tree, then the

Proportion of amino-acid differences between an outgroup (OU) and a human (HU), myomorph (MY) or guinea-pig (GP) sequence

Protein	OU-HU	OU-MY	OU-GP
Outgroup: marsupial			
α -crystallin A	0.10	0.09	0.09
α -globin	0.19	0.23	0.25
β -globin	0.26	0.31	0.30
α -lactalbumin	0.46	0.55	0.55
pancreatic ribonuclease	0.34	0.34	0.38
Outgroup: bird			
lipoprotein lipase	0.23	0.23	0.27
lipocortin	0.28	0.32	0.28
insulin	0.38	0.41	0.51
nerve growth factor- β	0.42	0.47	0.43
Outgroup: factor X			
factor IX	0.63	0.61	0.63

- Ginsberg, J. R. & Macdonald, D. W. *IUCN, World Conservation Union*, Gland, Switzerland (1990).
- Scott, J. *Painted Wolves* (Hamish Hamilton, London, 1991).
- Fuller, T. K. & Kat, P. W. *Afr. J. Ecol.* **28**, 330–350 (1990).
- Gascoyne, S. C. *et al. J. wildl. Dis.* (in the press).
- Carey, A. B. & McLean, R. G. *J. appl. Ecol.* **20**, 777–800 (1983).
- van Heerden, J. & Kuhn, F. S. *Afr. J. wildl. Res.* **15**, 80–84 (1985).
- Kaplan, C. (ed.) in *Rabies The Facts* 1–21 (Oxford University Press, 1977).
- May, R. M. *Nature* **320**, 13–14 (1986).
- Soave, O. A. *Am. J. Vet. Res.* **25**, 268–269 (1964).
- Haig, D. A. in *Rabies The Facts* (ed. Kaplan, C.) 53–69 (Oxford University Press, 1977).

ML method supports the true tree only for the group of nonconservative proteins but not for the group of conservative proteins. This would not be a good property. It is more reasonable to argue that tree III is the true tree and the ML method supports this tree for the group of conservative proteins.

In conclusion, there is actually no conflict between the result of Hasegawa *et al.* and ours, because when the more divergent sequences are excluded their analysis also supports our hypothesis. But as we have noted¹, more sequence data are required to resolve

whether rodents are polyphyletic or whether our analysis represents a dramatic example that unequal rates of evolution can consistently mislead parsimony inference.

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1. Graur, D., Hide, W. A. & Li, W.-H. *Nature* **351**, 649–652 (1991).
2. Hasegawa, M., Cao, Y., Adachi, J. & Yano, T.-A. *Nature* **355**, 595 (1992).
3. Li, W.-H. & Graur, D. *Fundamentals of Molecular Evolution* (Sinauer, Sunderland, Massachusetts, 1991).

No Palaeocene 'mammal-like reptile'

SIR — Fox *et al.*¹ believe that a tooth-bearing fragment of a dentary and four isolated teeth of a distinctive new taxon, *Chronoperates paradoxus*, from the Palaeocene of Alberta, Canada, extend the record of 'mammal-like reptiles' (or, properly speaking, nonmammalian synapsids) by some 100 million years. A review of the anatomical evidence at hand does not bear out their remarkable claim.

Fox *et al.* enumerate four features in support of their interpretation of *Chronoperates* as a nonmammalian cynodont: (1) single-rooted lower postcanines with transversely narrow, multiple-cusped crowns lacking cingula; (2) presence of pseudoprismatic enamel; (3) retention of postdentary bones including a splenial; (4) small masseteric fossa.

First, it should be pointed out that the postcanine teeth are, in fact, quite distinct from those of derived Triassic nonmammalian cynodonts such as *Microconodon* mentioned by Fox *et al.* In *Microconodon*^{2,3} and the closely

related *Pseudotricodon*⁴, the multiple-cusped postcanines typically have at least incipiently divided roots. Furthermore, these teeth have anteroposteriorly aligned, rather than obtusely angled, cusps, and lack the peculiar interlocking of crowns found in *Chronoperates* (and similarly in various mammalian taxa). The derived absence of cingula is a character of doubtful phylogenetic significance⁴; cingula are also absent or at best slightly developed in the early Jurassic *Sinoconodon*, which is considered the most primitive known mammal by many authors^{5,6}.

Second, the phylogenetic significance of pseudoprismatic ultrastructure of the enamel has been the subject of continuing debate. Recent work indicates that most Mesozoic mammals (or mammaliaforms) have pseudoprismatic or 'preprismatic' enamel⁷.

Third, the alleged 'posteromedial trough' is rather different from the trough for the postdentary bones (articular, prearticular, surangular, angular) on the dentaries of nonmammalian cynodonts (see figure) and primitive mammals and is more likely to represent the posterior entrance of the mandibular canal. Significantly, *Chronoperates* lacks the internal mandibular groove for the more anterior portion of Meckel's cartilage (the posterior portion being represented by the articular bone) found in nonmammalian cynodonts and primitive mammals⁸. The 'scar for splenial and prearticular' is quite unlike the corresponding features on the lingual surface of the dentaries of nonmammalian cynodonts (see figure). There is no feature on any known dentaries of undoubted nonmammalian cynodonts that can be homologized with the 'hook-shaped depression' (which might be a

preservational artefact).

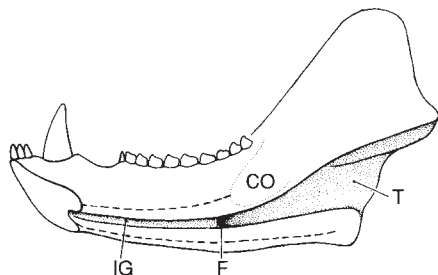
Finally, the full extent of the masseteric fossa cannot be determined on the holotype of *C. paradoxus* owing to the fragmentary condition of the coronoid process, but the masseteric fossa in all advanced nonmammalian cynodonts is as extensive as in mammals⁹.

The fossils currently available do not justify classification of *Chronoperates* as a nonmammalian cynodont and the resultant range extension of about 100 million years for nonmammalian synapsids. *Chronoperates* shares no clearly derived characters with any known taxon of nonmammalian cynodonts^{5,10}, and Novacek¹¹ noted that the dental differences between this form and symmetrodont mammals are rather subtle. There is a clear need for more complete cranial material to determine the precise affinities of this interesting new taxon.

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1. Fox, R. C., Youzwyshyn, G. P. & Krause, D. W. *Nature* **358**, 233–235 (1992).
2. Simpson, G. G. *Am. J. Sci.* **12**, 87–108 (1926).
3. Sues, H.-D., Olsen, P. E. & Kroehler, P. A. in *In the Shadow of the Dinosaurs: Early Mesozoic Tetrapods* (eds Fraser, N. C. & Sues, H.-D.) (Columbia University Press, New York, in the press).
4. Hahn, G., Lepage, J. C. & Wouters, G. *Bull. Soc. belges Géol.* **93**, 357–373 (1984).
5. Hopson, J. A. in *Origins of the Higher Groups of Tetrapods* (eds Schultze, H.-P. & Trueb, L.) (Cornell University Press, Ithaca, 1991).
6. Luo, Z. & Crompton, A. W. in *Mammal Phylogeny Vol. 1* (eds Szalay, F. S., Novacek, M. J. & McKenna, M. C.) (Karger, Zürich, in the press).
7. Carlson, S. J. in *Skeletal Biomineralization: Patterns, Processes and Evolutionary Trends Vol. 1* (ed. Carter, J. G.) 531–556 (Van Nostrand Reinhold, New York, 1990).
8. Krebs, B. in *Early Mammals* (eds Kermack, D. M. & Kermack, K. A.) 89–102 (Academic, London, 1971).
9. Barghusen, H. R. *Postilla* **116**, 1–49 (1968).
10. Hopson, J. A. & Barghusen, H. R. in *The Ecology and Biology of Mammal-like Reptiles* (eds Hotton, N., MacLean, P. D., Roth, J. J. & Roth, E. C.) 83–106 (Smithsonian Institution, Washington DC, 1986).
11. Novacek, M. J. *Nature* **358**, 192 (1992).
12. Crompton, A. W. *Proc. zool. Soc. Lond.* **140**, 697–753 (1961).



Dentary of the derived nonmammalian cynodont *Diademodon* (based on ref. 12) in lingual view to show the trough for the postdentary bones (T), posterior foramen for the mandibular canal (F) and articular facets ('scars') for the splenial (broken lines) and coronoid (CO). Note forward continuation of the trough as internal groove (IG).

Scientific Correspondence

Scientific Correspondence is a relatively informal section of *Nature* in which matters of general scientific interest, not necessarily those arising from papers appearing in *Nature*, are published. Because there is space to print only a small proportion of the letters received, priority is usually given according to general interest and topicality, to contributions of fewer than 500 words, and to contributions using simple language.

A trot through time

Patrick Cunningham

Horse Power: A History of the Horse and the Donkey in Human Societies. By Juliet Clutton-Brock. *Harvard University Press/Natural History Museum*: 1992. Pp. 192. \$29.95, £19.95.

IN AD 441, the Huns came boiling out of Asia, galloping west and south to begin the dismantlement of the Roman provinces. They were neither the first nor last great eruption from this region. From the Scythians through Ghengis Khan to the Tartars of more recent memory, this broad belt of grassland ecosystems, stretching from the Ukraine to China, has regularly sent out mounted hordes to remake history.

Horse power made possible these great convulsions of human society. Of all the 4,000 or so mammalian species still in existence, *Equus caballus* is probably the only one on which these great movements could have taken place. Humans can ride on elephants, asses, camels, buffaloes and cattle. The horse, however, has some especially useful structural features. Evolution in open grassland has produced a herbivore that can survive on any passing vegetation. The horse is fast (the single-digit hoof is a help) and good at sustained work, because continuous digestion in the caecum does not need to be interrupted with long phases of rest for rumination.

Horse Power is the very appropriate title chosen by Juliet Clutton-Brock for her fascinating history of the horse (and its asinine cousins) from prehistory to last year's Derby. With a substantial record of investigation and publication in animal evolution and ethology, she brings a scientist's insight to the often contentious areas of evolution, archaeology and the history of human societies. At the same time, technical fundamentals of anatomy, physiology and the mechanics of horse tackle are documented. A pleasing aspect of the book, as she leads us through the often conflicting theories, is the combination of a scientist's detached assessment of the facts and a historian's appreciation of the place for inference, analogy and balanced rationalization. The result is a smoothly flowing text.

The plan, logically enough, is chronological. Horses evolved in Eurasia, and a reasonable speculation (for it can be no more) says that the heart of their range would have been somewhere north of the Caspian Sea. They spread across the Eurasian landmass and the Americas in prehistoric times, but did not exist in Africa. In America they became extinct, and were reintroduced five centuries

ago. In Africa, they were also introduced by humans. Domesticated asses, however, have an African origin. The wild ass of Asia (the onager, also known as the half-ass) is not their ancestor. Doubts on this score have been laid to rest by differences in chromosome numbers and by cross-breeding studies which

at a million dollars a time?

The final chapters deal with the fascinating story of how the Plains Indians in North America built a new lifestyle on horseback, and how the white man, again on horseback, brought the Indians down to earth once more; of how the Agricultural Revolution in northern Europe, which made the Industrial Revolution possible, was again horse powered; of the horse in war; and finally of today's leading entertainment industry, the racing business.

Given the amount of ground covered, it is inevitable that the book ends in a breathless gallop. Perhaps for the same reason, hooves seem to touch the ground mainly on English soil. Indeed, enthu-

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J. Wootton's painting of the Byerley Turk (c. 1700), one of three Arabian stallions from which all modern racehorses and thoroughbreds are descended.

show that asses can be successfully crossed with the African wild ass, but not with surviving onagers.

In this broad sweep of equine history, there are some curiosities. Why did the stirrup escape invention until mediaeval times? Why did the horse as a draught animal not become popular until recent centuries, and then only in northern Europe? Was it simply because of the ease with which a yoke sits on the bullock's neck? What is the genetic basis for the extraordinary character of the ass-horse hybrid, the mule, and its reciprocal, the hinny?

Clutton-Brock has done a marvellous job of assembling illustrations along the way — from the fascinating grave goods from the frozen tombs of Pazyryk in Siberia, to Eclipse ("first, and the rest nowhere"). But where is Northern Dancer, who died in 1990 at the age of 26 after a career siring offspring

siam for the home team produces some partisan viewpoints: Blucher would be miffed to read that the charge of the Household Brigade under Lord Uxbridge decided the Battle of Waterloo, and the ladies of Bayeux would no doubt resist the suggestion that their famous tapestry is a product from across the English Channel.

These cavils apart, it is clear that Clutton-Brock has succeeded in producing a book with the substance and authority to satisfy specialists in the field, and which can still catch and hold the interest of the general reader. □

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E. T. Archive

Animist debate

Mark Ridley

The New Anthropomorphism. By J. S. Kennedy. Cambridge University Press: 1992. Pp. 194. £27.95, \$54.95 (hbk); £10.95, \$17.95 (pbk).

ANTHROPOMORPHISM means explaining the behaviour of nonhuman animals by the kind of mental processes that we attribute to humans, and it is generally thought to have been killed a century ago by Lloyd Morgan's canon and the rise of behaviourism. But according to John Kennedy, "anthropomorphism remains much more of a problem than most of today's neobehaviourists believe".

The core of Kennedy's short, provocative, readable and (in my opinion) ultimately unconvincing book "consists of nineteen essays which appear to be erroneous and can be traced to unwitting anthropomorphism". The ideas come from all parts of ethology — they include animal suffering, cognition, hierarchical control, intentionality, migration (particularly R. Baker's work on it) and motivation — and the book reads like an argument with almost everyone in the field. It is mainly British ethologists, and their extended family, who are put in the pillory; but Kennedy has fetched a few outsiders too, such as the students of ape language. It says much for the well-mannered tone of the book that, although Kennedy specifically criticizes many people, it is unlikely that any of them will take personal offence at what he says; I do doubt, however, whether they will agree with him.

The errors that Kennedy detects are of two main kinds: from behavioural ecology and from cognitive ethology. The anthropomorphism of behavioural ecology is mainly what Kennedy calls "mock anthropomorphism" and he has no serious objection to it; it often helps, when applying the theory of natural selection, to imagine that animals, or even genes, make strategic decisions. The errors arise, Kennedy suggests, when behavioural ecologists confuse ultimate causes with proximate ones, and suppose that animals actually do calculate costs and benefits. But there is room for argument about where the confusion lies. When I read the behavioural ecologists quoted by Kennedy, I rarely found the same interest in proximate mechanisms as he did; to me they sounded more like statements about adaptation, perhaps marred by a slip of the (maybe lazy) pen. He objects to the idea of 'warning' signals, for example: "Describing an animal's action as a warning to its fellows effectively takes it for granted that



A male panther chameleon *Chamaeleo pardalis*, one of the most hostile and territorial chameleons, threatens his reflection in a mirror; when unaroused, most of his body would be dark green. The picture is taken from *Chameleons: Nature's Masters of Disguise* by J. Martin and A. Wolfe. Published by Blandford, £16.99.

those actions were intentional on the part of the animal. Intentions are proximate causes of behaviour, so again we have an ultimate, functional cause masquerading as a proximate one in the animal." Oh no we don't! We merely have a signal that is given in an objectively recognizable set of circumstances (danger) and that has predictable consequences in the behaviour of other animals nearby (they take cover). No intention has either been "taken", or mistaken, "for granted".

In cognitive ethology, various anthropomorphic experiences have been suggested to reside in animals, including consciousness, language and suffering. Kennedy argues that there is no good evidence for any of them. So far, so good, but the conclusions he draws are exaggerated. Having shown, for example, that the evidence for consciousness in nonhumans is unconvincing, he concludes, "altogether, then, it seems likely that consciousness, feelings, thoughts, purposes, etc., are unique to our species and unlikely that animals are conscious". This is argumentative agnosticism sliding into atheism.

Moreover, Kennedy's sceptical argument itself does more damage than he allows. It works rather well against humans. He argues that mock anthropomorphism works in behavioural ecology because "natural selection has produced animals that act *as if* they had minds like us", but denies that success is evidence for anthropomorphic mechanisms. So why not for humans too? Humans, it is true, do provide linguistic assurances as well as observable behaviour, but is not

language logically only observed, in the form of 'verbal behaviour', in the same way as physical movement? Surely it is rampant animism to postulate more than a simple neuronal circuit in people who are behaving verbally, as well as physically, 'as if' they are suffering when you stick an electrode in them. Kennedy criticizes researchers on animal suffering because "they have no adequate evidence that animals do actually suffer"; but that is too strong a demand. Nor, for that matter, does he have any evidence that animals do not suffer. Suffering needs only to be a plausible approximate assumption (like optimization, in behavioural ecology) to stimulate research.

For the fate of anthropomorphism, like that of the physiological methods Kennedy prefers, will not be settled by pronouncements from the armchair. It will be settled in the laboratory and in the field. D. Cheney and R. M. Seyfarth (as quoted by Kennedy) claim that "anthropomorphizing *works*"; Kennedy refers to "the blind alley of neoanthropomorphic cognitivism". We shall see. If anthropomorphism produces results that are, in the normal manner of science, valuable, it will be persisted with; if it does not, it will be abandoned. It was, after all, abandoned once before. The only danger, Kennedy might reply, is that scientists can be enduringly obstinate in their investigations of blind alleys. Just think how long behaviourism lasted in psychology. □

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Critical days

David Shoenberg

Superconductivity: Its Historical Roots and Development from Mercury to the Ceramic Oxides. By Per Fridtjof Dahl. *American Institute of Physics*: 1992. Pp. 406. \$55.

ALTHOUGH superconductivity was discovered more than 80 years ago, it is only during the past 35 years or so that it has been fundamentally understood. And even this understanding has recently proved to be incomplete with the discovery five years ago of new materials that remain superconducting to high temperatures — a discovery that has brought about an almost explosive revival of activity in the field owing to the exciting technical implications. The history of how all this has come about has, however, received relatively little attention, so Per Fridtjof Dahl's book is to be welcomed, particularly because the bulk of it deals with the now rather forgotten early years, before the breakthrough made in 1956 with the fundamental 'BCS' theory of J. Bardeen, L. N. Cooper and J. R. Schrieffer.

The first 20 years of this period, which might be called the 'pre-history', is usually taken for granted, but here it takes up more than two-thirds of the book and is much enlivened by extracts from original sources such as the archives of the principal participants. One interesting sidelight is a discussion of G. Holst's role in the discovery of superconductivity. It looks as if Kamerlingh Onnes may not have given sufficient credit to the very capable but junior colleague who carried out crucial measurements. Dahl goes even further back and devotes more than 50 pages to the 'pre-pre-history', the early history of the liquefaction of gases, culminating in Onnes's liquefaction of helium in 1908 and the even earlier studies on electrical resistance. This is indeed a fascinating story, with colourful personalities such as J. Dewar, W. Ramsay, K. S. Olszewski, Z. F. von Wroblewski and their feuds and rivalries, but it has already been eloquently described in K. Mendelssohn's *Quest for the Absolute Zero* (Taylor and Francis, 1977) and is somewhat peripheral to a history of superconductivity.

During the 15 years between Onnes's discovery of superconductivity in 1911 and his death in 1926, he had a virtual monopoly in following up his discovery,

because the liquid-helium facilities he had created in Leiden were unique until the last years of his life. By today's standards, progress during those years was gradual, but not because Onnes was leisurely. On the contrary, he drove his colleagues hard. Dahl tells how, at Onnes's funeral, his laboratory assistants walking behind the hearse had to run as the vehicle speeded up to make up for a delay and one said to another: "The old devil — even after he has gone he makes us run." The reason progress was slow was partly the lack of competition, so that Onnes sometimes spent too much time following up details that proved to be unimportant, partly because of his

brought out well by Dahl in many long quotations from the "Leiden Communications" and from conference reports. With hindsight, the discussion at the Solvay conference in 1924 seems particularly muddled. Onnes's health was already failing by then and it was W. H. Keesom who presented a long report on superconductivity and replied to comments in the lively discussion that followed. One of the issues was the applicability of thermodynamics to the destruction of superconductivity by a magnetic field. P. W. Bridgeman argued that thermodynamics was indeed applicable, whereas Keesom disagreed because he took it as obvious that the transition was irreversible. In fact, of course, reversibility was eventually justified with the discovery of the Meissner effect in 1933; but it was a weird formula that Bridgeman produced for the field dependence of the critical field, which he did by integrating the analogue of the Clapeyron–Clausius equation. Without any real justification, he assumed that the latent heat of transition varied as the fourth power of the temperature and that the difference of magnetic susceptibility in the normal and superconducting states obeyed Curie's law and varied as the inverse of the temperature. Nevertheless, he could claim that his final formula for the temperature dependence of the critical magnetic field was in reasonable accord with the scanty data then available. Strangely, Keesom did not comment on the absurdity of Bridgeman's assumptions (nor did any of the other eminent physicists present), but he pointed out that when superconductivity (which he assumed simply meant infinite conductivity) is destroyed by a magnetic field, the magnetization cycle, completed after reducing the field to zero again, must be irreversible. But although Keesom explicitly speaks of a "locked-in" flux after the cycle is completed, the illustrative diagram in the text of the conference discussion mysteriously shows no locked-in magnetization. This error might of course have occurred in the publishing process, but it is odd that Dahl should reproduce the erroneous diagram (and also Bridgeman's erroneous formula) without any comment. It is indeed a general defect of Dahl's presentation that he tends to reproduce extracts from original sources without adequate critical comment or explanation.

Over the 25 years or so between Onnes's death and the Second World War, and even more so for about 10

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Kamerlingh Onnes: discoverer of superconductivity in 1911.

famous philosophy of *door meten tot weten* (through measurement to knowledge), which led to too much emphasis on measurement and too little on interpretation. But probably most important was the lack of sufficient theoretical understanding of the basic theory of metals. Indeed, superconductivity remained a baffling phenomenon even after quantum mechanics had brought much clarification. This was epitomized by Felix Bloch's 'theorem' in the 1930s that 'there is no theory of superconductivity that cannot be refuted'.

The confused state of understanding is

years after the war, the pace of activity quickened. More and more laboratories set up facilities to experiment at liquid-helium temperatures, and the development of quantum mechanics provided a better understanding of solid-state theory. Dahl charts this progress with particularly thorough treatment of the history of one of the most important landmarks, the Meissner effect, by reference to many original papers from the Meissner and other archives. Further landmarks were the phenomenological theory of the brothers Fritz and Heinz London, the experimental study of the small penetration depth of a magnetic field into a superconductor, the study of how superconductors react to high-frequency electromagnetic fields, and the isotope effect, which was the final clue leading to the violation of Bloch's 'theorem' and the successful development of the BCS theory. All this is well presented, but it is a pity that Dahl omits altogether the discovery of Josephson tunnelling, one of the most important landmarks since the BCS theory. This omission is strange in view of the technical potentialities of the Josephson effect and of Dahl's emphasis in the rest of the book on other technical applications of superconductivity, particularly magnet technology.

The rest of the book is devoted mostly to superconducting magnets and their application to the development of ever larger accelerators for studying elementary particle physics. This topic is introduced by an interesting account of the development of the special 'type II' superconducting alloys from which high-field magnets can be wound. But the chapter on how the magnets can be used in the design of accelerators is far from clear to the uninitiated. It assumes far too much specialized knowledge, not only of machine design but also of the devious politics of the struggles between the proponents of the various rival schemes. Perhaps it is because this is Dahl's own field that he assumes that his labyrinth of acronyms and technical jargon will be transparent to others.

The book is somewhat marred by misprints and misspellings of names, usually merely irritating to a pedantic reader, but occasionally actually misleading, such as in the attribution of work by Lorenz to Lorentz (although the name index does correctly distinguish between them). Dahl is also sometimes fallible when there is a Russian context.

He might well be forgiven for not knowing that L. V. Shubnikov, a pioneer of type II superconductivity, was executed in 1937 rather than dying from heart failure in 1943, as this information was revealed only in 1992. But his puzzlement and speculation as to why A. A. Abrikosov should not have referred to Shubnikov's work until after 1957 is a little naïve. He credits me with a three-year stay in Moscow (it was actually a year), quoting E. L. Andronikashvili's amusing but not altogether reliable autobiography, and he refers more than once to Landau's Institute, when in fact he means Kapitza's Institute for Physical Problems (of which Landau and his group formed a part); the Landau Institute came into being only after Landau's death. And a final example: he speculates on why the publication of an impor-

tant letter to *Nature* from Shalnikov (in Kapitza's Institute) should have taken over a year to appear in print. In fact, the letter, which I myself put into English, was submitted on 27 April 1938 and appeared on 9 July 1938.

But despite its shortcomings, the book will be found to be a good read not only by those, like myself, who have sentimental memories of the early days, but also by those who have entered the field more recently and would like to know something of the personalities and the events behind the tidied-up results that they are likely to meet today. So do read it, but read with caution and beware of accepting everything as gospel. □

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Zeroing in on the cold

A. J. Leggett

Low Temperature Physics: An Introduction for Scientists and Engineers. By P. V. E. McClintock, D. Meredith and J. K. Wigmore. Blackie: 1992. Pp. 296. £65.

THE intended readership of this book is well conveyed by its subtitle: people who have a reasonable general background in physics or a related subject at the level, say, of an undergraduate degree in the United Kingdom or first-year graduate course in the United States, but little previous acquaintance with low-temperature physics as such. After an introduction which, *inter alia*, emphasizes the general importance of low temperatures and previews some of the more striking phenomena peculiar to this regime, the authors give accounts of the low-temperature behaviour of normal crystalline solids, superconductors (including the recently discovered copper oxide materials, which, with transition temperatures up to 125 K, strain the traditional definition of 'low' temperature phenomena somewhat), superfluid helium-4 and the normal and superfluid phases of liquid helium-3. The book is completed by a chapter on experimental methods at low temperatures and one on applications (chiefly the use of superconductors to generate high currents and magnetic fields, and Josephson electronics).

The treatment is largely descriptive, and the authors for the most part quote theoretical results without detailed derivation. The exception is the chapter on superfluid helium-4, where they derive both the phenomenon of Bose condensation and the propagation of first and second sound in some detail. (Given

this, I was a little surprised that in the chapter on superconductivity they did not find it worthwhile to reproduce Cooper's simple argument on the formation of a bound state in a Fermi system with arbitrarily weak attraction, which is certainly no more complicated.) Generally, the account, qualitative as it necessarily is, of current theoretical ideas about superconductivity, superfluidity and so on is clear and up to date (although I personally regret the emphasis, in the explanation of superfluidity in general, on the so-called Landau criterion, which in my opinion explains the critical velocity observed for ions in superfluid helium and very little else).

I have a few minor criticisms: although the exposition is in general careful and readable, there are a few places where the text is liable to confuse or mislead the reader (such as in the discussion of phonon thermal conductivity on page 47, and of magnetic resonance in superfluid ³He, on pages 207–8), and in adapting figures from the published literature the authors have not always taken sufficient care to make the notation consistent (Fig. 4.2) or the caption self-contained (Fig. 2.4). But on the whole, the book will serve its intended readership well. □

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Correction

In B. Oakley's review of *Hard Drive* (*Nature* 359, 25; 1992), the artist Vanessa Bell, a member of the legendary Bloomsbury Group, is mistakenly credited as President Roosevelt's science adviser, and with the idea of the personal computer, instead, of course, of Vannevar Bush.

New Journals Issue

Nature's New Journals supplement, in which more than 50 publications will be assessed, will appear in next week's issue (1 October).

Observation and possible causes of new ozone depletion in Antarctica in 1991

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Local ozone reductions approaching 50% in magnitude were observed during the Antarctic spring in the 11–13 and 25–30 km altitude regions over South Pole and McMurdo Stations in 1991. These reductions, at altitudes where depletion has not been observed previously, resulted in a late September total ozone column 10–15% lower than previous years. The added depletion in the lower stratosphere was observed to coincide with penetration into the polar vortex of highly enhanced concentrations of aerosol particles from volcanic activity in 1991.

ON the basis of the phase of the quasi-biennial oscillation (QBO) of equatorial stratospheric winds¹, and tropical Pacific sea surface temperatures (SST)^{2,3}, which affect the timing of transport of ozone to the Antarctic continent in spring, 1991 was expected to be a year of early recovery of the springtime Antarctic ozone hole and only moderate ozone depletion. Although the recovery of the ozone hole indeed occurred early in 1991 (mid-November), total ozone nevertheless reached record lows in September even though values during the October minimum period at South Pole were no lower than the previous record low values observed in 1987. Antarctic ozone depletion generally extends over the altitude range from 12 to 22 km (ref. 4), the dominant region of polar stratospheric cloud (PSC) formation. From balloon-borne observations, which give the vertical ozone profile, we have determined that this 'normal' ozone hole was only slightly more severe during September in 1991 than in 1987, 1989 or 1990; additional ozone depletion was observed, however, in both the 11–13 km and the 25–30 km regions which were the predominant causes of the low total ozone at this time. Homogeneous chlorine chemistry, combined with continually increasing chlorine levels, can explain only a small portion of the high-altitude ozone depletion. The transport of coastal air depleted of ozone by PSCs and/or the aerosol cloud from the volcanic eruption of Mount Pinatubo at 10° N in June 1991 might also be involved. The low-altitude ozone reduction occurs simultaneously with high aerosol concentrations, from the August 1991 eruption of Mount Hudson at 46° S, arriving in the Antarctic lower stratosphere in September, and is believed to be the result of heterogeneous processes occurring on the surface of the volcanic aerosol.

Observations

Ozone vertical profiles were obtained at South Pole and McMurdo (78° S) stations with a digital electrochemical ozonesonde technique which has been used in Antarctica since 1986 (ref. 4). Total ozone is obtained by integration of the vertical profiles of ozone partial pressure for soundings that reach at least 28 km. Beginning on 10 October, there was adequate sunlight to obtain total ozone values with the Dobson spectrophotometer at South Pole station (W. Komhyr, personal communication) and the sonde values agreed with the Dobson values within ~2%.

As indicated in Fig. 1, a minimum in total ozone of about 125 Dobson units (DU) was observed at South Pole station on 7 October (Julian day 280). Although this value is not significantly lower than the lowest value observed there in 1987, the September reduction began earlier in 1991 than in any

previous year. In late August 1991, total ozone was similar to previous years with a value of ~270 DU on day 240 (28 August); but beginning on day 247 (4 September), six of the next seven measurements were ~30–40 DU (15–20%) lower than observed in the previous five years. The 'normal' ozone hole, represented by the 12–20 km column data in Fig. 1, contributed only about 1/4 of the difference in total ozone between 1991 and previous years during the September decreasing phase. Thus, in terms of total column ozone, 3/4 of the unusual low ozone (~20–30 DU or ~10% of the late August column) during September had to result from new ozone losses in regions not previously subject to depletion.

Individual profiles of the ozone mixing ratio for all soundings at South Pole between 15 August and 3 November, shown in Fig. 2, indicate that high-altitude (25–30 km) ozone reductions, 30–50% below the normally constant mixing ratio in this region, began on 4 September and were observed to some extent on every sounding within the vortex thereafter. Because of the position of the high-altitude ozone minima coinciding with the maximum altitude obtained on 4 and 9 September, the extrapolation for total ozone was done at a lower-than-normal mixing ratio and thus the total ozone values for these days are probably underestimated.

Transport of mid-latitude ozone to the south polar region at high altitudes, as expected during vortex breakup in late October

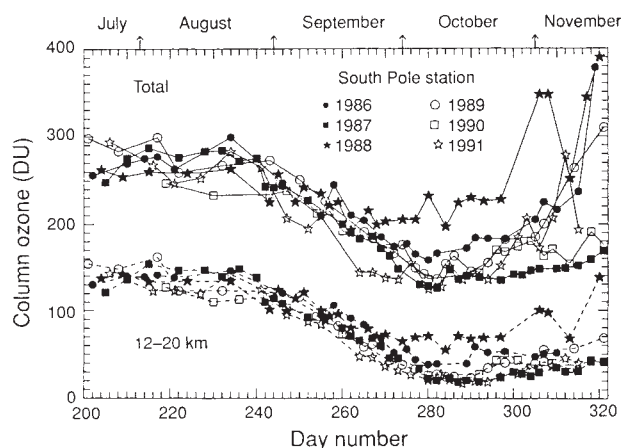


FIG. 1 Total and 12–20 km column ozone against time, obtained from integration of ozonesonde profiles at South Pole Station for the past six Antarctic spring seasons.

and early November, is also apparent in Fig. 2. Beginning about 5 November, the vortex distorted and shifted substantially towards east Antarctica so that ozone concentrations typical of extra-vortex air were present over the South Pole down to ~ 23 km (see ozone profile of November 8 in Fig. 2). The vortex centre then shifted back towards the South Pole and the ozone profile on 11 November again showed the high-altitude minimum, indicating that it was a characteristic feature of the vortex.

Ozone soundings were conducted at McMurdo between 23 August and 1 November in 1991. Figure 3 compares the 1991 McMurdo and South Pole soundings in September (Fig. 3a) and October (Fig. 3b) with those obtained in 1990. Soundings from McMurdo when the vortex boundary was near, for example from 13 to 21 September 1991, are identified by the high values of ozone mixing ratio at high altitude in Fig. 3. Ozone minima in the 27–30 km region, similar to those observed at South Pole, were present during soundings conducted while McMurdo was inside the vortex boundary in 1991, providing important confirmation that this ozone feature extended over much and possibly all of the vortex. Figure 3b indicates that small ($\sim 10\%$) ozone minima were present at 25–28 km in October at both South Pole and McMurdo before 1991.

Another unusual ozone depletion phenomenon, observed in 1991, is demonstrated in Fig. 4 at South Pole station where mixing ratios at 10, 12 and 14 km averaged over 2-km intervals are compared with measurements of the previous five years. The small ozone layer below the main ozone hole, which in the past

has been characterized by mixing ratios of ~ 0.3 – 0.4 parts per 10^6 volume (p.p.m.v.) in the 11–13 km region, began to decrease in late September and reached values 50% below normal in late October. The time constant for ozone reduction was ~ 40 days, about one-half as fast as the ozone hole development at higher altitude in September. Similar observations were made at McMurdo.

Possible causes of the new depletion

The layered nature of the high-altitude ozone reductions suggests that they may be related to transport processes. Although the upper stratosphere was dynamically active during the period in question, quasi-horizontal transport from a region having ozone mixing ratios as low as 2 p.p.m.v. in the 27–30 km region is highly unlikely because ozone mixing ratios are larger at lower latitudes in this altitude region. To account for these low ozone values, downward motions would have to originate at 50–55 km, begin in early September and be unusually severe in 1991. Thus, the high-altitude ozone minima seem to represent an ozone loss. Unusually low ozone in the 11–13 km region can, in principle, result from upward motions of tropospheric air of low ozone content. For example, the unusually low ozone in the 11–15 km region on day 264 (21 September) in Fig. 4 was caused by a 2-km upward motion in association with a major vortex perturbation. Such motions were not, however, present thereafter. Similarly, transport from the tropics, where this altitude region would be in the troposphere, could account for the unusually low ozone.

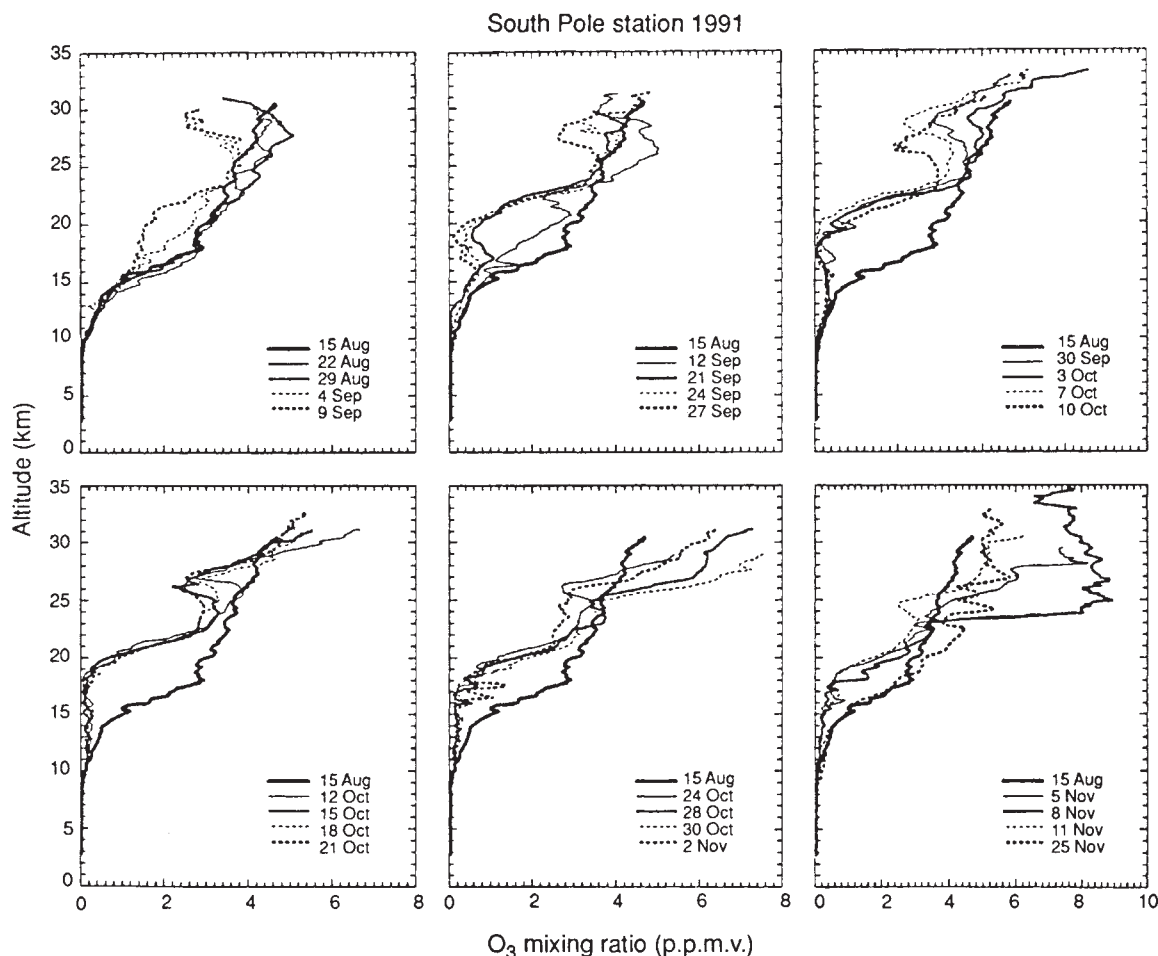


FIG. 2 Vertical profiles of ozone mixing ratio obtained at South Pole station between 15 August and 25 November 1991, showing development of the high-altitude ozone reduction in the 25–30 km region.

Study of isentropic back trajectories from South Pole on the 330–340 K (~ 12 km altitude) and 700–800 K (26–28 km) surfaces, using the European Centre for Medium-range Weather Forecasting gridded analysis, reveals the following. In the 12 km region, the trajectories during the low-ozone period are generally confined to the vortex, verifying that the phenomenon is peculiar to the vortex at this time. At 26–28 km, all days that indicated an ozone deficit in this region had trajectories which reached the coast in 1–2 days, generally in the Wedell Sea area. Those days with minimal or no ozone deficits had trajectories generally over east Antarctica in the 3–4 days before arriving at South Pole. Those with high ozone values, indicating extra-vortex air, had trajectories which extended north of 60° S, as expected. These calculations suggest that the source of the low ozone values at high altitude is in the coastal Wedell Sea/Palmer Peninsula area and thus could be related to PSCs which may have been generated by mountain lee waves in this region earlier. But such activity would have to have been much stronger in 1991 than in previous years.

Current theory of springtime Antarctic ozone depletion requires the presence of PSCs, consisting of both ice and nitric acid trihydrate (NAT) particles, for the heterogeneous chemical conversion of inactive chlorine-bearing molecules into active forms and for the suppression of reactive nitrogen (see Solomon⁵ for a review of these processes). Temperatures at South Pole reached minima of about -90°C in the 20–24 km region from mid-July to mid-August in 1991 and were below the frost point during this period at altitudes below ~ 24 km, and below the NAT existence temperature below ~ 26 km, for maximum pre-winter H_2O and HNO_3 vapour mixing ratios of 5 p.p.m.v. and 10 p.p.b.v. (parts per 10^9 volume), respectively. Thus, PSCs composed of ice or NAT would not have formed above these altitudes. Satellite limb-scanning extinction measurements cannot observe PSCs poleward of $\sim 80^\circ$ latitude, but lidar observations at South Pole (G. Fiocco, personal communication) and

backscatter sondes⁶ flown to 25 km on several occasions during the winter of 1991 at South Pole did not reveal the presence of extensive PSCs above the typical 12–22 km altitude range (J. Rosen, personal communication). It may be possible that there were unusually low temperatures at 25–30 km over other locations in Antarctica and that transport brought air depleted in ozone to the South Pole and McMurdo at these altitudes. An unusual PSC-related source for the high-altitude ozone reductions therefore cannot be ruled out.

Because ozone removal at 30 km may occur through homogeneous chemistry, we reinvestigated gas-phase ozone removal over South Pole for 1990 conditions and compared them to 1980, using a photochemical model⁷. Preliminary calculations indicate that for 1990 chlorine concentrations, homogeneous chemistry results in at most a 10% reduction in ozone at 30 km compared with 1980. Thus, for the high-altitude ozone losses observed in 1991, at most one-quarter of the deficit could be caused by gas-phase chemistry. The smaller reductions observed at high altitude in October 1990, however, may have been the result of such a process.

The new ozone losses observed in Antarctica in 1991 may also be the result of heterogeneous chemistry on particle surfaces other than those presented by PSCs. The observation that ozone is decreasing in the lower stratosphere at a rate faster than expected for homogeneous chlorine-related processes has led to considerable interest in whether the global stratospheric aerosol layer, composed of aqueous $\text{H}_2\text{SO}_4/\text{H}_2\text{O}$ droplets, can affect ozone through heterogeneous processes. Eruptions of Mount Pinatubo in the Philippine Islands on 15–16 June 1991 resulted in a highly enhanced aerosol layer, predominantly in the 22–26 km region, which was at least as large as that observed after the eruption of El Chichón and probably larger⁸. Within six weeks of the eruption, over 95% of the Pinatubo aerosol particles that appeared at northern mid-latitudes were aqueous $\text{H}_2\text{SO}_4/\text{H}_2\text{O}$ droplets^{9,10}. During the period 12–15 August,

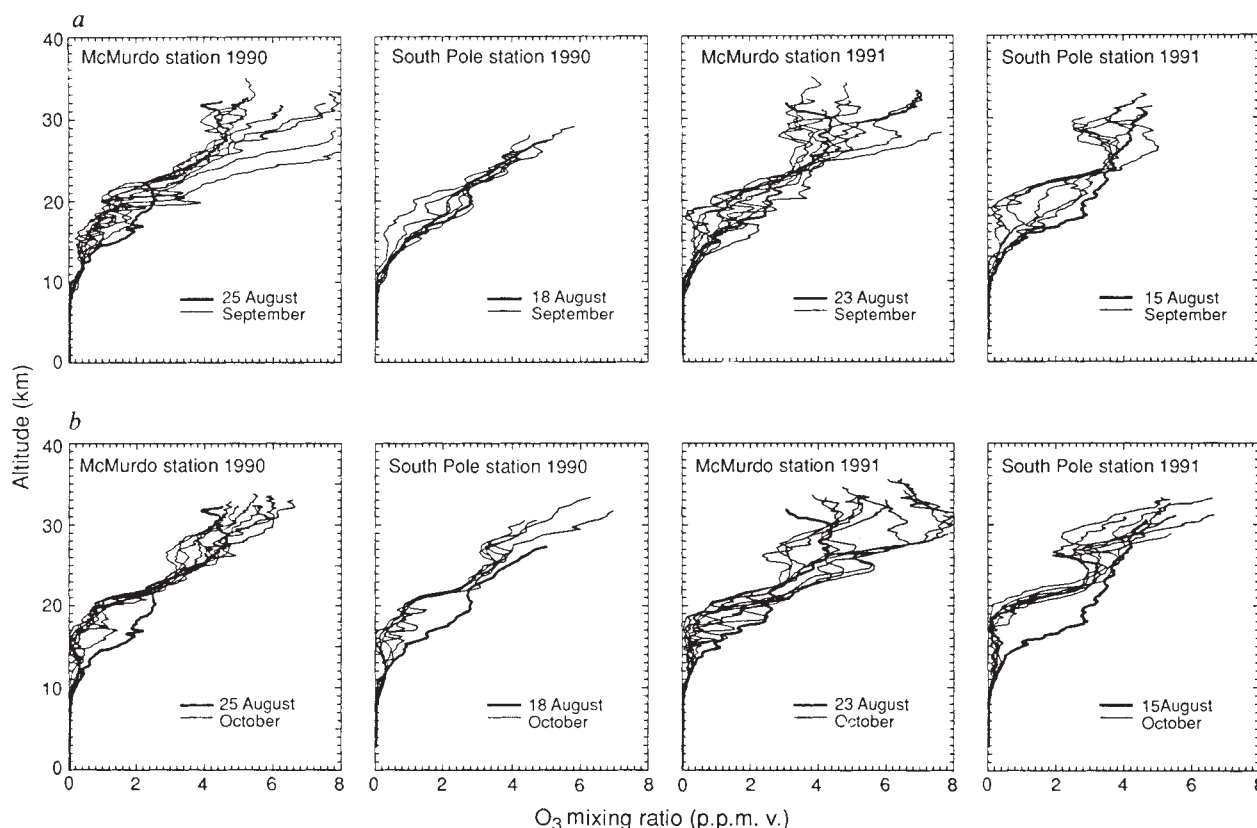


FIG. 3 Comparison of ozone mixing ratio profiles in 1990 and 1991 at South Pole and McMurdo stations in (a) September and (b) October. The heavy curves are the ozone profiles before development of the ozone hole.

Mount Hudson (46° S) in Chile erupted, injecting an SO₂ cloud into the lower stratosphere⁸.

On the basis of measurements of the total surface area and H₂SO₄ fraction of the stratospheric aerosol after the 1982 eruption of El Chichón, and the heterogeneous reaction probabilities of N₂O₅ and ClONO₂ with H₂O on the surface of the volcanic aerosol, Hofmann and Solomon¹¹ estimated that ozone reductions of ~15% could have occurred in the 20 km altitude region at northern mid-latitudes during the winter of 1982–83. These results are in agreement with subsequent model studies¹² and observations¹³ and it is generally believed that volcanic aerosols are capable of perturbing the stratospheric ozone layer. The perturbation is enhanced at low temperatures because the water content of the aerosol increases, increasing the probability of ClONO₂ reacting with H₂O on the surface thereby leading, in the presence of sunlight, to the formation of free chlorine, as occurs in PSC-related reactions.

There is a considerable body of evidence that volcanic aerosol was efficiently transported towards the pole in the Southern Hemisphere stratosphere in mid-1991. The SAGE II satellite aerosol extinction instrument¹⁴, which made observations to 50° S, indicated substantial penetration of the Pinatubo cloud to high latitudes in the Southern Hemisphere, especially that portion of the cloud between 21 and 40 km, beginning in mid-July. The Hudson eruption cloud was transported in a southeasterly direction and encircled the Antarctic vortex region below 16 km by September⁸. Lidar measurements at Aspendale, Australia (38° S)¹⁵, detected a strong backscatter layer from the Hudson eruption at 12–14 km on 28 August. This layer, in a region where there was essentially no aerosol from the Pinatubo eruption, gave an aerosol backscatter signal more than 5 times that of Pinatubo which had been observed in the 16–26 km region after mid-July.

Column NO₂ measurements at Lauder, New Zealand (45° S), indicated NO₂ depletion beginning in mid-August and reaching a peak of at least 25% below normal in the total column by mid-October¹⁶. This reduction is likely to be due to heterogeneous processes on the surface of volcanic aerosol particles, probably involving the reaction of N₂O₅ and H₂O. A similar effect was observed at northern midlatitudes during the winter of 1982–83, following the eruption of El Chichón¹¹.

The presence of high reactive chlorine, combined with sunlight, results in ozone depletion below 20 km as early as mid-August in the 68° S region¹⁷, where solar illumination begins earlier than at the pole. Air that had been exposed to the Pinatubo aerosol and had already undergone substantial heterogeneous chemical processing probably surrounded the Antarctic vortex to altitudes in excess of 20 km by mid-August. This suggests that the stratosphere near the Antarctic coast was primed for ozone depletion above the altitudes typically subjected to such loss (12–20 km). The fact that the high-altitude ozone minima progressed to lower altitude with time (see Fig. 2) could be interpreted as being due to subsidence of air depleted in ozone which had been transported into the high-altitude vortex in early September. If this were the case, one would expect volcanic aerosol to accompany the low-ozone air.

Lidar observations at South Pole (G. Fiocco, personal communication) do not indicate the presence of enhanced aerosol in late August and early September above 15 km but clearly show high-altitude layers in November. The lidar at Dumont d'Urville (68° S) saw an apparently dense volcanic particle layer at 20–23 km on 20 July and more frequent volcanic layers up to 26 km during September, generally when the station was outside of the vortex (L. Stefanutti, personal communication). Optical depth measurements at South Pole station (E. Dutton, personal communication) indicate excessive aerosol present on 10 October, the first day with adequate sunlight for measurement. The optical depth reached values in excess of 0.3 in November, more than 3 times higher than during the corresponding period following the eruption of El Chichón and similar to values

observed for the Pinatubo eruption in the main equatorial layer⁸. Thus, the observations suggest the absence of volcanic aerosol at high altitude in the vortex and its presence in the coastal region in September.

Balloonborne particle counter measurements to altitudes of 27 km and lidar measurements¹⁸ were made at McMurdo between 23 August and 13 October, and although no excess aerosol was detected above 20 km, volcanic particle layers were observed in the lower stratosphere as early as 11 September. Stormy weather from 13–23 September, coinciding with a substantial compression of the vortex in the McMurdo region where the polar jet maximum appeared overhead, prevented observations during this period. Large enhancements of aerosol in the 10–14 km region were observed after the September storm and thereafter until the final measurements in mid-October, during periods when McMurdo was well within the vortex¹⁸. Concentrations of newly nucleated small aerosol (radii ~0.01 µm) greater than 6,000 cm⁻³ were observed in the volcanic cloud. At these high concentrations, the lifetime for coagulation of these condensation nuclei is only a few days so that the source of the condensing vapours was clearly the Hudson eruption, rather than Pinatubo. As indicated earlier, Pinatubo had little or no effect below about 15 km. Above this altitude, condensation nuclei from the Pinatubo eruption had returned to normal levels by September¹⁸. The data at McMurdo thus provide strong evidence that the low ozone at 12 km was not the result of unusual intrusions of tropical air but was associated with the volcanic cloud from Mount Hudson.

There was no evidence of temperature increases in this layer that might be the result of aerosol heating and cause vertical motions of the air parcel and thus ozone reductions. At 12 km, the air is 10 times as dense as at 25 km where changes in circulation may take place in response to heating in dense volcanic layers at high altitude in equatorial regions. In fact, modelling of the heating effect of the Pinatubo cloud at 16–28 km predicts upward motions in the equatorial region and downward

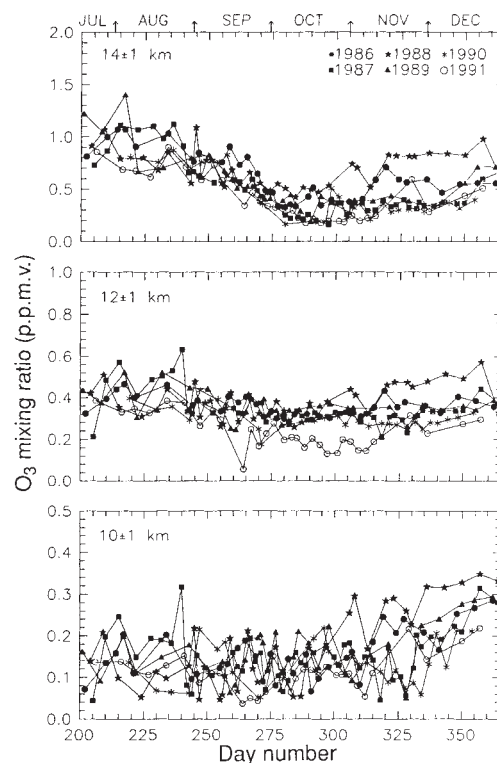


FIG. 4 Two-kilometre averaged ozone mixing ratios at 10, 12 and 14 km each austral spring from 1986 to 1991 at South Pole station.

motions at mid-latitudes, which would tend to decrease and increase ozone, respectively, in these regions¹⁹. Thus, if any vertical motion were expected south of 30°, it would be downward with increasing ozone.

The total aerosol surface-area concentration in the Hudson cloud, due mainly to the many freshly nucleated small particles, peaked at 12 km with values as high as $100 \mu\text{m}^2 \text{cm}^{-3}$, enough to cause substantial heterogeneous chemical perturbations¹¹. Temperatures ranged from -68°C at the bottom to -75°C at the top of the layer, too warm for PSCs but dictating a water content of $\sim 40\%$ in the $\text{H}_2\text{SO}_4/\text{H}_2\text{O}$ aerosol and thus a relatively high probability for the production of free chlorine through the reaction of ClONO_2 and H_2O on the surface of the aerosol (reaction probability ~ 0.003)²⁰. But unless the aerosol were frozen, it is unlikely that surface reactions of ClONO_2 with HCl , as occurs on ice and NAT particles, would be important. Thus, one would expect the rate of ozone reduction due to volcanic aerosol at these temperatures to be slower than in the case of PSCs. The observation of a slower depletion rate in the volcanic layer than in the normal ozone hole conforms with this expectation. Modelling of volcanic ozone depletion rates²¹, although done for higher altitudes, indicates that for this level of volcanic aerosol surface area and reaction probability, ozone should decline at a rate about one-half that experienced in the normal ozone hole region, in agreement with observations.

In summary, loss of half of the Antarctic ozone in the 11–13 km layer during October in 1991 (see Fig. 4) occurs simultaneously with penetration into the vortex of enhanced volcanic aerosol below 15 km. The most plausible explanation of this ozone loss seems to be depletion occurring through heterogeneous chemistry in the volcanic cloud.

Implications for the 1992 Antarctic ozone hole

Concentrations of volcanic $\text{H}_2\text{SO}_4/\text{H}_2\text{O}$ aerosol at least 3 times as large as those present after El Chichón have now been incorporated into the 1992 winter vortex. At -80°C , the volcanic aerosol particles will have a water content similar to NAT particles ($\sim 50\%$) but with a total mass possibly 10 times greater. If the volcanic hypothesis proposed here to explain the low ozone at 12 km in 1991 is correct, then ozone reductions could occur during 1992 as early as May, especially in the 70° to 80°S latitude band where low levels of sunlight coincide with cold temperatures at this time.

The concentration of effective condensation sites for the formation of PSC particles will also be increased during the winter of 1992. This will result in the formation of more numerous but smaller PSC particles for the same amount of condensable mass, increasing the surface area available for heterogeneous reactions and increasing PSC lifetimes. Both ice crystals and NAT particles appear at very low concentrations ($\sim 0.001\text{--}0.005 \text{cm}^{-3}$)²² and thus are believed to form selectively on the larger sulphate aerosol particles (radius $\geq 0.3 \mu\text{m}$)²³. Initial measurements of the Pinatubo aerosol size distribution indicate a stratospheric enhancement of the larger particles by up to two orders of

magnitude⁹. For constant condensable mass, particle surface area varies as the $1/3$ power of the concentration so that an increase in PSC particle surface area, and possibly heterogeneous chemical effects, from a factor of 2 to a factor of 4 can be expected. Increasing the surface area of PSCs may not substantially decrease the minimum total ozone observed because depletion in the heart of the ozone hole is not limited by available surface area during a normal winter.

Smaller PSC particles have smaller sedimentation velocities and are therefore likely to yield decreased denitrification. The temporary sequestration of odd nitrogen in PSCs (generally referred to as denoxification) will, however, still take place when temperatures are low enough, so that active chlorine levels would remain high so long as PSCs are present. Termination of ozone depletion in the lower stratosphere may occur earlier under these conditions because springtime evaporation of the lingering smaller NAT particles will return HNO_3 to the gas phase to be photolysed. A change in the morphology of the ozone hole, including an earlier appearance and expansion of its vertical distribution, can be expected.

Summary and conclusions

Apart from the normal springtime ozone depletion over Antarctica, observed between 12 and 20 km in 1991, there were additional ozone losses of about 50%, compared to previous years, in the upper and lower stratosphere. The impact on total ozone loss was substantial (10–15%), being large enough to account for record lows in total ozone during late September at South Pole station in 1991 and to contribute towards the depth of the ozone minimum in early October. The upper stratospheric ozone loss occurred in the 25–30 km region, was observed to develop in early September and became a stable feature of the south polar vortex in 1991. About one-fifth of this loss seems to have been present in previous years and may be related to homogeneous chemistry. The remainder seems to be associated with transport of air depleted in ozone by heterogeneous processes.

Ozone losses of $\sim 50\%$ in the 11–13 km region began in late September. This ozone loss in the lower stratosphere is believed to be a new phenomenon peculiar to the 1991 polar vortex and its relationship to volcanic activity in 1991 has been examined. The presence and timing of what is believed to be aerosol from the Mount Hudson eruption at 11–14 km, coincident with the unusual ozone loss in this region at both South Pole and McMurdo, represents what we believe to be the strongest evidence for heterogeneous ozone loss in association with volcanic aerosol obtained to date. In 1992, volcanic aerosol will have been trapped in the winter vortex. The observations of new ozone depletion in the Antarctic lower stratosphere presented here, and the volcanic hypothesis proposed to explain it, predict that the volcanic aerosol could cause ozone depletion in Antarctica this austral fall as well as adding to the normal PSC-related springtime depletion. In addition, the volcanic aerosol particles, acting as condensation sites for PSC formation, are likely to alter the ozone hole morphology in 1992. □

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- Garcia, R. R. & Solomon, S. *Geophys. Res. Lett.* **14**, 848–851 (1987).
- Angell, J. K. *Geophys. Res. Lett.* **17**, 1569–1572 (1990).
- Komhyr, W. D., Oltmans, S. J., Grass, R. D. & Leonard, R. K. *Can. J. Phys.* **69**, 1093–1102 (1991).
- Hofmann, D. J., Harder, J. W., Rolf, S. R. & Rosen, J. M. *Nature* **326**, 59–62 (1987).
- Solomon, S. *Nature* **347**, 347–354 (1990).
- Rosen, J. M., Krome, N. T. & Oltmans, S. J. *Geophys. Res. Lett.* **18**, 171–174 (1991).
- Garcia, R. R. & Solomon, S. *J. geophys. Res.* **88**, 1379–1400 (1983).
- Smithsonian Institution Bull. *glob. Volcanism Network* **16**, No. 7, 8, 9, 10 (1991).
- Deshler, T., Hofmann, D. J., Johnson, B. J. & Rozier, W. R. *Geophys. Res. Lett.* **19**, 199–202 (1992).
- Sheridan, P. J., Schnell, R. C., Hofmann, D. J. & Deshler, T. *Geophys. Res. Lett.* **19**, 203–206 (1992).
- Hofmann, D. J. & Solomon, S. *J. geophys. Res.* **94**, 5029–5041 (1989).
- Brasseur, G. P., Granier, C. & Walters, S. *Nature* **348**, 626–628 (1990).
- Jäger, H. & Wege, K. *J. atmos. Chem.* **10**, 273–287 (1990).
- McCormick, M. P. & Veiga, R. E. *Geophys. Res. Lett.* **19**, 155–158 (1992).

- Barton, I. J., Prata, A. J., Watterson, I. G. & Young, S. A. *Geophys. Res. Lett.* **19**, 1211–1214 (1992).
- Johnston, P. V., McKenzie, R. L., Keys, J. G. & Matthews, W. A. *Geophys. Res. Lett.* **19**, 211–214 (1992).
- Tuck, A. F. *J. geophys. Res.* **94**, 11687–11737 (1989).
- Deshler, T. *et al. Geophys. Res. Lett.* (in the press).
- Brasseur, G. & Granier, C. *Science* (in the press).
- Hanson, D. R. & Ravishankara, A. R. *J. geophys. Res.* **96**, 17307–17314 (1991).
- Prather, M. J. *geophys. Res.* **97**, 10187–10191 (1992).
- Hofmann, D. J. & Deshler, T. *J. geophys. Res.* **96**, 2897–2912 (1991).
- Hofmann, D. J., Rosen, J. M., Harder, J. W. & Hereford, J. V. *J. geophys. Res.* **94**, 11253–11269 (1989).

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Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis

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The retinoblastoma gene, a prototypic tumour-suppressor gene, encodes a nuclear phosphoprotein (Rb). To understand better the role of Rb in development and in tumorigenesis, mice with an insertional mutation in exon 20 of the *Rb-1* locus were generated. Homozygous mutants die before the 16th embryonic day with multiple defects. The haematopoietic system is abnormal; there is a significant increase in the number of immature nucleated erythrocytes. In the nervous system, ectopic mitoses and massive cell death are found, particularly in the hindbrain. All spinal ganglion cells die, but the neural retina is unaffected. Transfer of the human retinoblastoma (*RB*) mini-transgene into the mutant mice corrects the developmental defects. Thus, Rb is essential for normal mouse development.

RETINOBLASTOMA, an ocular childhood tumour, has been a model for studies of the role of tumour suppressor genes in cancer predisposition^{1,2}. The hereditary form of the disease is an autosomal dominant trait³. But a recessive nature of the mutant gene was proposed in Knudson's 'two-hit' hypothesis⁴, and later substantiated⁵⁻⁹. Although the eye is usually the first site of tumour formation, patients with hereditary retinoblastoma have a high risk of developing additional neoplasms later in life. These tumours tend to be of unusual types such as osteosarcomas or soft-tissue sarcomas¹⁰.

The retinoblastoma (*RB*) gene was isolated by a positional cloning strategy⁷⁻⁹. Molecular analyses have shown that the gene is ubiquitously expressed and evolutionarily conserved in vertebrates^{9,11,12}. Studies of various tumours have revealed that diverse mutations, including deletions, duplications and point mutations, can inactivate the gene¹³. The gene is expressed in normal retina but transcripts are frequently absent or abnormal in retinoblastoma cells⁷⁻⁹, and recent studies have demonstrated that somatic mutation of both *RB* alleles occurs in many tumour types including breast carcinoma, prostate carcinoma, osteosarcoma, soft-tissue sarcoma, and small-cell lung carcinoma¹³. Introduction of the wild-type *RB* gene into Rb-deficient tumour cells suppresses their neoplastic phenotype¹⁴⁻¹⁸, suggesting that loss of Rb function may contribute significantly to tumorigenesis.

The *RB* gene encodes a nuclear protein (Rb)¹² that is a substrate of the *cdc2* kinase^{19,20}. Phosphorylation of Rb oscillates during the cell cycle; unphosphorylated Rb predominates in G1, whereas phosphorylated forms appear as cells enter S phase and persist during the G2 and M phases²¹⁻²⁵. Recent findings have suggested that Rb might be involved in the control of cell-cycle progression, because the injection of excess unphosphorylated Rb into cells in early G1 blocks their entrance into S phase²⁶. The discovery of stable protein-complexes between Rb and the oncogenic proteins of several DNA tumour viruses, such as the E1A protein of adenovirus, T antigen of simian virus 40 (SV40), and the E7 protein of papillomavirus²⁷⁻²⁹ inspired a search for cellular proteins that interact with Rb. Several such proteins were found, including c-Myc and N-Myc³⁰ and a cellular transcriptional factor, E2F³¹⁻³⁴. These Rb-associated proteins are themselves involved in cell-cycle regulation and all of them interact with Rb through T/E1A-binding domains³⁵⁻³⁷.

This region seems to be important for Rb function because carboxy-terminal truncations of Rb deleting the T/E1A-binding domains are nonfunctional^{16,38}.

Taken as a whole, the data surrounding the behaviour of Rb present something of a paradox. Its ubiquitous expression and seeming involvement in cell-cycle regulation suggest that it plays a central role in essential cellular activity. But by contrast, germ-line mutation of the *RB* gene in humans is strikingly specific in that it predisposes the individual to retinoblastoma with 90% penetrance³. To help resolve this paradox, we have created a mutation (*Rb-1^{Δ20}*) of the mouse *Rb-1* gene in a line of embryonic stem (ES) cells. Passage of this mutation into the mouse germ line (by the production of ES cell chimaeras) has shown that homozygous *Rb-1^{Δ20}/Rb-1^{Δ20}* mutants are not viable. They die in mid-to-late gestation with defects in the haematopoietic system as well as the central and peripheral nervous systems.

***Rb* gene targeting and Rb-deficient mouse lines**

To mutate the *Rb-1* locus in embryonic stem cells, a positive-negative selection strategy³⁹ was used to disrupt exon 20, a region that is frequently altered in tumours. The targeting vector, pMG5RB-neo-2TK, contained 8 kilobases (kb) of genomic *Rb-1* sequences encompassing exons 19 to 22. A *PGK-neo* expression cassette containing the neomycin resistance gene driven by the phosphoglycerate kinase promoter⁴⁰ served both to create an insertional mutation in exon 20 and to select positively for transfected cells (Fig. 1). Two viral thymidine kinase genes under the control of the pMC1 promoter were used to flank the *Rb-1* sequences and served to select against cells with a random integration of the targeting vector⁴¹.

The linearized targeting vector was electroporated into 1.7 × 10⁸ AB1 ES cells⁴². Simultaneous selection in G418 and FIAU yielded about 2,210 colonies, a 17-fold enhancement over G418 selection alone. A total of 850 colonies were analysed by Southern blotting for the presence of a specific gene-targeting fragment. The endogenous exons 19-23 are encoded on a 13.5 kb *HindIII* fragment. Because the inserted *neo* cassette also contains a *HindIII* site, a correctly targeted recombination is expected to generate two fragments instead of one (Fig. 1d). Initial screening of the ES cell clones using a 3' probe (Fig. 1e, probe A), identified several candidate clones with the expected 7.5 kb *HindIII* fragment. Additional restriction enzymes and different probes confirmed the initial identification. Both the 3' and the 5' probes gave the predicted restriction fragment lengths as did

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probes made from the *neo* gene itself (no hybridization to DNA from the original AB1 cells was observed when *neo* was used as a probe, Fig. 1f). This demonstrates that the expected recombination event had occurred at the *Rb-1* locus. We have called the mutant allele *Rb-1*^{Δ20} to denote the termination mutation in exon 20 of *Rb-1* by the insertion of *PGK-neo*. A total of 42 targeted clones were identified, representing a frequency of one targeted clone per 1.5×10^6 cells transfected.

Cells from 5 of the 42 clones were microinjected into 3.5-day C57BL/6 blastocysts. Four of the five clones gave rise to highly chimaeric mice (typical for the AB1 cells). As anticipated from

the degree of chimaerism, cells from these four clones contributed to gametogenesis in the chimaeras, as indicated by transmission of the agouti coat colour to their progenies in crosses with C57BL/6 females. Mouse lines established from two clones, 36-8 and 25-10, were used for subsequent analysis. No selection against *Rb-1*^{Δ20} gametes was found because a ratio of 1:1 heterozygous and wild-type progeny was seen in crosses between heterozygous and wild-type mice. Surprisingly, no retinoblastoma was found in more than 100 heterozygous mice examined. In contrast, continuous observation has led to the recent identification of brain tumours in mice of ages 4, 7, 8

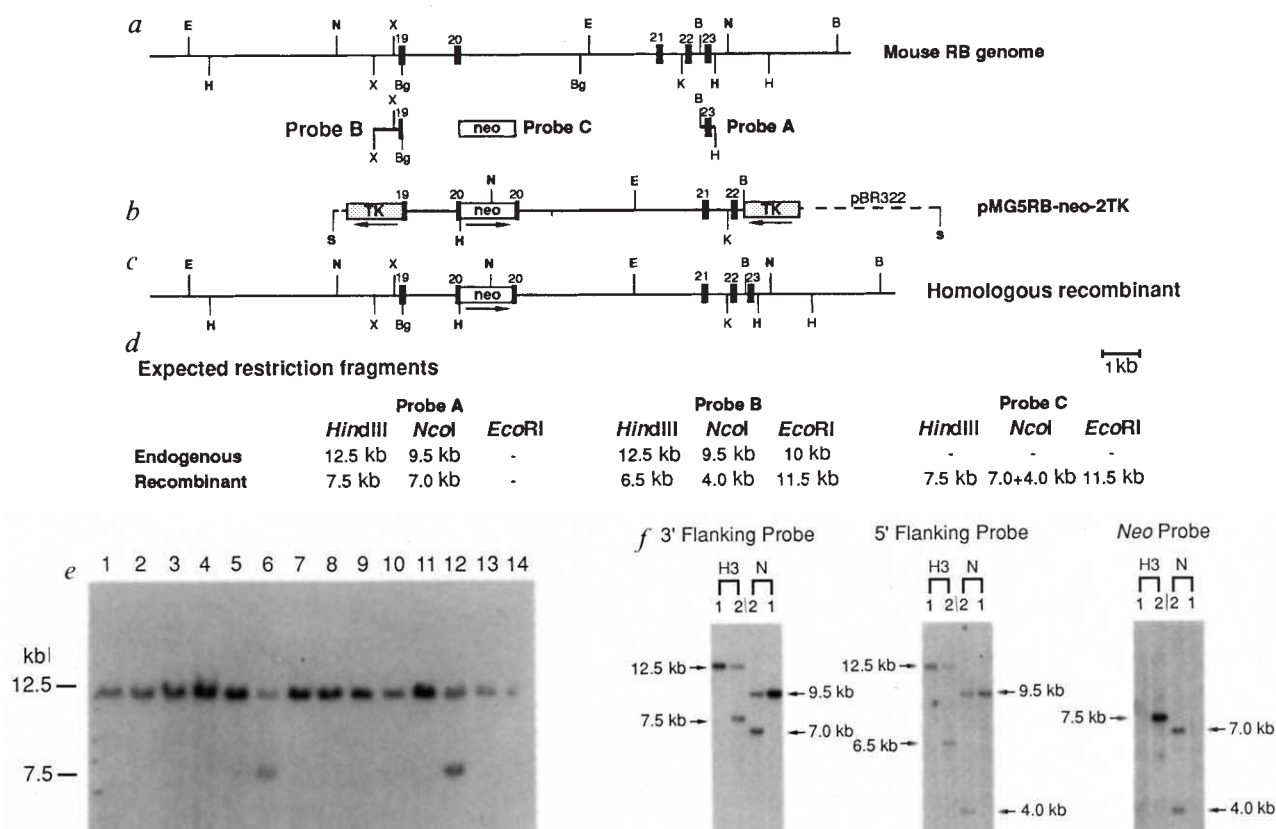


FIG. 1 Disruption of the *Rb-1* gene in mouse embryonic stem cells. **a**, Restriction map of the mouse genomic *Rb-1* fragment. An 8 kb genomic DNA fragment encompassing exons 19 to 22 is illustrated. **b**, Restriction map of the targeting construct, pMG5RB-neo-2TK. A *neo* cassette was inserted into exon 20; and the genomic fragment was flanked by TK cassettes. **c**, The predicted structure of a mutated allele after homologous recombination. The probes used for identification of allele-specific recombination are shown. **d**, Expected sizes of various restriction fragments detected by the flanking and neomycin probes. **e**, Initial screen of the targeted *Rb-1* clones by Southern blot hybridization analysis. The *HindIII* 7.5 kb restriction fragments indicate the expected recombinant allele. **f**, Confirmation of the targeted disruption. DNA samples from untransfected cells (1) and one candidate recombinant clone (2) were digested with *HindIII* (H3) or *NcoI* (N), and probed with either one of the flanking probes or *neo*. An additional fragment of the expected size is found in the recombinant clone in each hybridization. Abbreviations: E, *EcoRI*; B, *BamHI*; Bg, *BglII*; H, *HindIII*; K, *KpnI*; N, *NcoI*; S, *SalI*; X, *XbaI*.

METHODS. The murine genomic *Rb-1* DNA fragment encompassing introns 18 to 23 was isolated from a 129/J genomic library. An 8.0 kb genomic DNA fragment extending from a *BglII* site in exon 19 to a *BamHI* site in intron 22 was subcloned into a plasmid vector for the construction of the targeting vector, pMG5RB-neo-2TK. The plasmid PGK-neo-bpA, containing a phosphoglycerate kinase I promoter and a bovine growth hormone polyadenylation sequence⁴⁸, was used for the neomycin cassette. Briefly, a *XhoI* fragment was excised from the PGK-neo-bpA plasmid, blunt-ended, and inserted into an *SstI* site in exon 20 of *Rb-1* genomic DNA fragment after the site was destroyed. To aid the construction, two *SstI* sites in intron 19 and 20 and one *BglII* site in intron 20 were removed by filling in with Klenow

enzyme. Insertion of the neomycin cassette into exon 20 would disrupt the coding sequence of the *Rb-1* gene. For negative selection, the plasmid, pBTK2, containing the *tk* gene of herpes simplex virus under the MCI polyoma enhancer and promoter was made by cloning an *EcoRI* fragment (446-628) of pMCI neopA into an *EcoRI* site of pHSV-tk. To make the two-*tk* cassette plasmid (pBR2TK), the *BamHI* site of pBTK2 was first blunt-ended, then an *XhoI*-*SalI* fragment was subcloned into *SalI* site of pBTK2. Finally, the *BglII*-*BamHI* fragment containing *Rb-1* genomic DNA sequences and the neomycin cassette was subcloned into the unique *BamHI* site of pBR2TK. The resulting construct contains two *tk* cassettes with opposite transcriptional orientation, one at each end of the *Rb-1* genomic sequences. The unique *SalI* site in the targeting construct, pMG5RB-neo-2TK, allowed the plasmid to be linearized at the 5' end. The AB1 cell line used in transfection was derived from an agouti 129/SvEv mouse⁴². Culture and transfection of the ES cells were as described⁴². Colonies were picked after 10-12 days of selection and cells were dissociated in droplets of trypsin. Each colony was then plated to a 24-well plate. Cells were trypsinized 7 days later and transferred to two sets of 24-well plates. One set of cells was frozen after reaching confluence, and genomic DNA was extracted from the second set of cells. About 10 µg of DNA was digested with *HindIII*, fractionated by electrophoresis through 0.8% agarose gels, transferred to Hybond nylon membrane (Amersham), and hybridized with probe A. Probe A, used in the initial screen of the candidate recombinant clones, is a *BamHI* and *HindIII* fragment containing exon 23. For further confirmation, probe B, an *XbaI* and *BglII* 5' flanking fragment, and an *neo* fragment were used. Transfer, hybridization and washing conditions were according to the manufacturer's recommendations with final washes in 0.1 × SSC at 60 °C for 15 min.

and 12 months. A second mutation removing the remaining wild-type *Rb-1* allele was found in these tumours (data not shown). These results are in agreement with ref. 43.

To determine the nature of any peptides made by the mutant gene, primary fibroblast cultures were established from wild-type, heterozygous and homozygous embryos at 11.5 days of gestation (Fig. 2a). Rb protein of M_r 105K could be detected on western blots of wild-type and heterozygous fibroblasts but

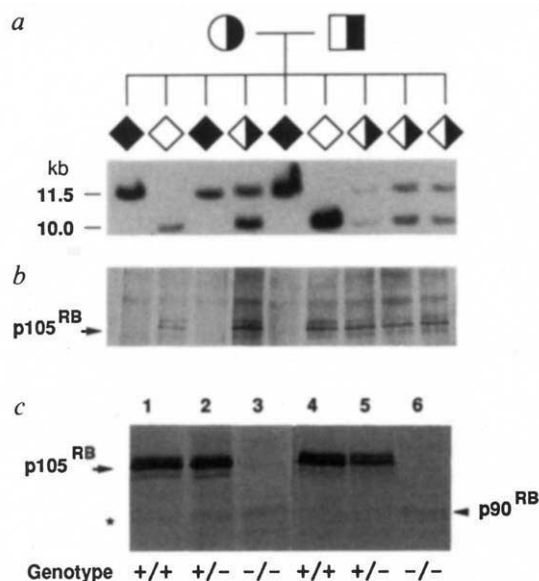


FIG. 2 Genotype identification and Rb protein expression of embryos from mating between heterozygotes. *a*, Southern blot analysis of *Eco*RI-digested DNA isolated from fibroblast cultures derived from a litter of nine E11.5 embryos. The upper 11.5 kb fragment represents the disrupted *Rb-1*^{Δ20} allele; and the lower 10.0 kb fragment is the wild-type *Rb-1* allele. Half-filled symbols indicate heterozygous genotypes at the *Rb-1* locus. Filled symbols represent the *Rb-1*^{Δ20}/*Rb-1*^{Δ20} mutant. The sex of the embryos was not determined and is therefore shown as a diamond symbol. *b*, Western blot analysis of Rb protein expression in fibroblast cultures derived from the embryos shown in *a*. The arrow indicates the unphosphorylated form of the full-length Rb protein. *c*, Identification of truncated Rb protein from homozygous embryos and fibroblasts by western blot analysis. Protein lysates (5 mg) from wild-type (lane 1), 10 mg each from heterozygote and homozygous mutant embryos (lanes 2 and 3) were used for immunoprecipitation. For the cultured fibroblasts, 20 mg protein lysates were used for immunoprecipitation in each lane (lanes 4–6). Truncated Rb proteins encoded by the mutant allele are shown both in the embryos and in the cultured fibroblasts. A nonspecific protein band (asterisk) is seen in all lysates prepared from embryos.

METHODS. To genotype mice, DNA was prepared from tails at the time of weaning (3 weeks of age) and analysed as described above for ES clones. To prepare primary fibroblasts, embryos were removed and dissected under sterile conditions. The embryos were briefly rinsed in PBS and then homogenized by passing them through a 25-gauge needle. The cell suspension and cell clumps were plated to a 10-cm tissue culture dish precoated with 0.1% gelatin. Cells were cultured in DMEM supplemented with 10% fetal calf serum. Fibroblast DNA was extracted and analysed as described for ES clones. For Rb protein analysis, mouse embryos were removed and quick-frozen in liquid nitrogen. The frozen tissue was homogenized in a cold mortar, and the tissue powder subsequently lysed in EBC buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40 containing 200 U ml⁻¹ aprotinin, 50 μg ml⁻¹ leupeptin and 1 mM phenylmethylsulphonyl fluoride). Cultured fibroblasts were washed with PBS, and lysed in lysis buffer on ice for 10 min. Protein concentration in the lysates was quantitated by the Bradford method⁴⁹. Monoclonal antibody Mab245, recognizing amino-acids 371–390 of exon 11 and 12 (ref. 50) was used for immunoprecipitation and immunoblotting as described²² (*b*). *c*, Mab4H6 (Canjia Inc., La Jolla, CA) recognizing epitope within exon 1–10 was used for immunoprecipitation and Mab245 for immunoblotting. Similar results were obtained using Mab4G2, a monoclonal antibody against the N-terminal region of Rb, (Canjia Inc., data not shown).

not in homozygous fibroblasts (Fig. 2b). Note that in addition to the prominent band of unphosphorylated Rb, a faint upper band of phosphorylated Rb protein is also seen. Insertion of the *neo* cassette would create termination codons in the *Rb-1* coding sequences and presumably lead to the synthesis of a C-terminal truncated protein. To test this, three monoclonal antibodies, specific for the amino terminus of Rb, were used in immunoprecipitation and western blotting. Low levels of a 90K protein were found in heterozygous and homozygous embryos (Fig. 2c, lanes 1–3), and fibroblast cultures (Fig. 2c, lanes 4–6). Note that an additional protein band of 86K, presumably the degraded product, was also seen. These truncated Rb proteins are very unstable as indicated by their low abundance in comparison with the amount of full-length Rb protein in heterozygotes.

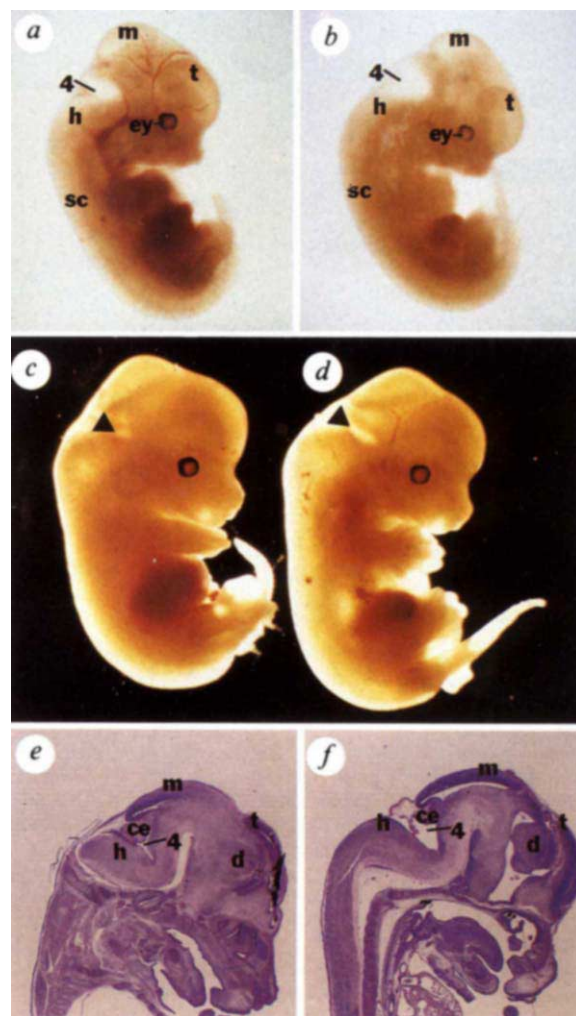


FIG. 3 Phenotypes of E12.5, and E13.5 embryos from a *+/-Rb-1*^{Δ20} × *+/-Rb-1*^{Δ20} cross. *a*, *b*, E12.5 embryos. A wild-type embryo is shown in *a* and an *Rb-1*^{Δ20}/*Rb-1*^{Δ20} embryo in *b*. *c*, *d*, E13.5 embryos. The wild-type embryo is shown in *c* and the mutant in *d*. The arrow indicates the location of the enlarged fourth ventricle. *e*, *f*, Sagittal views of E14.5 embryos of both wild-type (*e*) and *Rb-1*^{Δ20}/*Rb-1*^{Δ20} (*f*) genotype. Each pair of photographs (*a* and *b*, *c* and *d*, *e* and *f*) was taken at the same magnification. Note the pale colour of the mutant embryos. *d*, Diencephalon; *ey*, eye; *h*, hindbrain; *m*, mesencephalon; *t*, telencephalon; *4*, fourth ventricle; *sc*, spinal cord. Photographs of the embryos were taken on a Nikon SMZ-10 stereomicroscope with Fuji RD135 (ASA50) film immediately after dissection. For *e*, *f*, embryos were fixed in paraformaldehyde, embedded in paraffin, sectioned, and stained with haematoxylin and eosin according to standard procedures.

TABLE 1 Abnormality of embryos from $+ / Rb-1^{A20} \times + / Rb-1^{A20}$ crosses

Age (days)	Total conceptuses	Total normal embryos (%)	Genotype of normal embryos*			Total abnormal embryos (%)	Genotype abnormal embryos*			Phenotypically abnormal -/- embryos (%)
			+/+	+/-	-/-		+/+	+/-	-/-	
10.5	73	69 (95%)	17	28	20	4 (5%)	—	—	—	—
11.5	30	26 (87%)	12	8	5	4 (13%)	0	2	2	2/7 (29%)
12.5	60	43 (72%)	15	23	4	17 (28%)	0	0	9	9/13 (69%)
13.5	61	43 (70%)	10	29	3	18 (30%)	0	0	11	11/14 (79%)
14.5	28	21 (75%)	5	16	0	7 (25%)	0	0	6	6/6 (100%)
15.5	34	22 (65%)	5	17	0	12 (35%)	0	0	7	7/7 (100%)
16.5	18	16 (89%)	6	10	0	2 (11%)	0	0	1	1/1 (100%)

* +/+, +/- and -/- refer to embryos wild-type, heterozygous, and homozygous mutant at the *Rb-1* locus.

FIG. 4 Neuronal abnormalities in the *Rb-1^{A20}/Rb-1^{A20}* embryos. *a*, Coronal views of the hindbrain of an E12.5 mutant embryo. *b*, Closer view of the boxed area of *a* in which cell death (large arrowhead) in the intermediate zone and abundant mitotic cells in the ventricular zone (arrow) and in the intermediate zone (small arrowhead) can be found. *c-f*, Ectopic mitosis and neuronal cell death in the spinal cord and spinal ganglia of mutant embryos. Compared with the normal spinal ganglia of E15.5 (*c*), the ganglia in the mutant embryo are reduced as indicated by the arrow in (*d*). Cell deaths as indicated by their pycnotic remnants (arrow) are prominent in spinal ganglia (indicated of *g*) from an E13.5 mutant embryo (*e*). This observation probably explains the diminished size of the ganglia found in E15.5 embryos. Ectopic mitosis is observed in the intermediate zone of spinal cord of an E12.5 mutant embryo (*f*). Prominent mitotic figures in the ventricle zone (arrow) as well as in the migratory and differentiation zones (small arrowhead) are indicated, and cell death (large arrowhead) is also found in the migratory zone. Inset shows a closer view of the boxed area in which mitotic figure (small arrowhead) is found. Inferior olive from E15.5 wild-type (*g* and *i*) and mutant (*h* and *j*) embryos. Deregulated proliferation in the inferior olive (IO) is shown in the mutant embryos. *i, j*, Close-up views of the IO. Embryos were processed as described in Fig. 3. Photomicrographs of the embryos were taken on a Zeiss Axiphot microscope with Kodak Ektar 25 film.

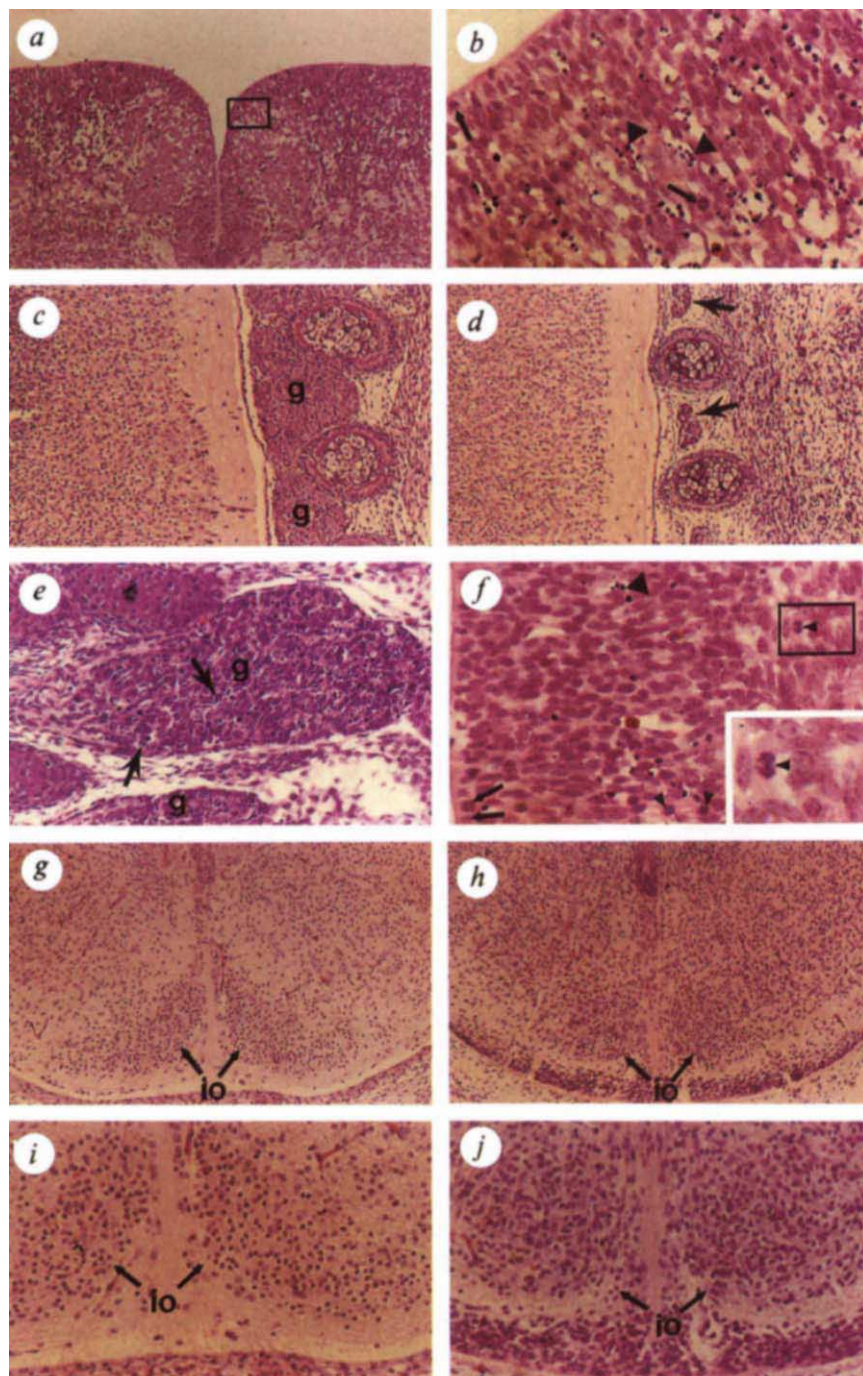
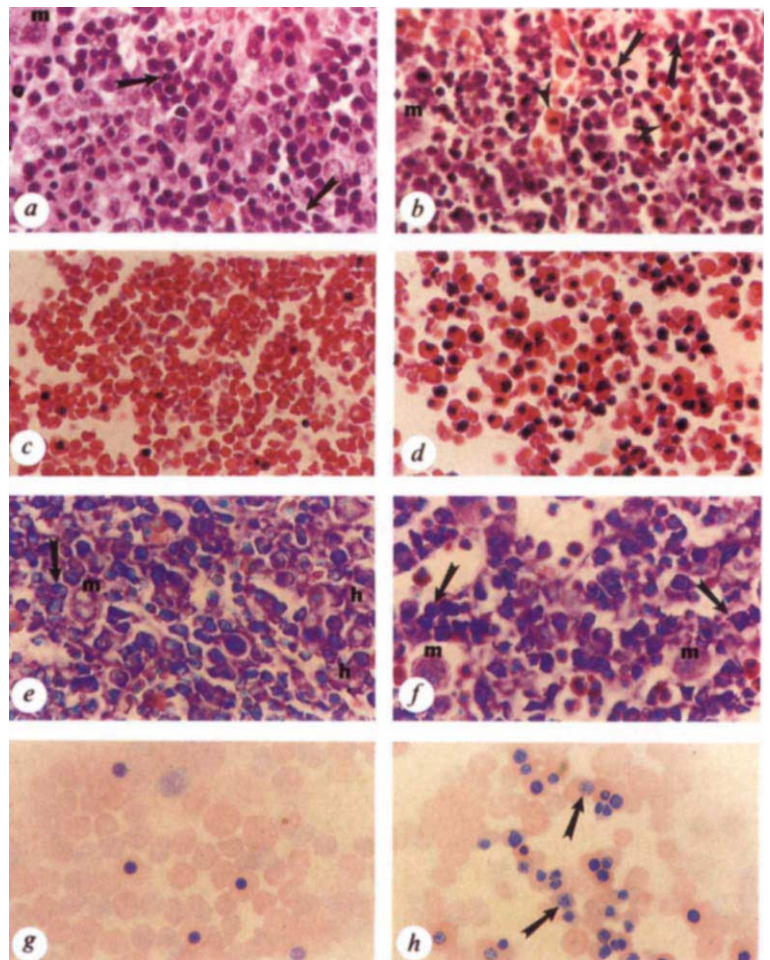


FIG. 5 Abnormalities of haematopoietic system in the $Rb-1^{\Delta 20}/Rb-1^{\Delta 20}$ embryos. Liver sections and peripheral blood cells from wild-type (left panel) and mutant (right panel) E15.5 and E14.5 (e, f) embryos are compared. Liver sections (a, b, e, f) and peripheral blood (c, d, g, h) were stained either with haematoxylin and eosin (a, b, c, d) or with Wright-Giemsa staining (e, f, g, h). In liver sections, hepatic haematopoiesis islands (arrow), nucleated erythrocytes (arrowhead), and megakaryocytes (m) are indicated. The enlarged sinusoids, fewer hepatocytes and predominance of nucleated erythrocytes are major features of the mutant liver. In peripheral blood, an abnormal ratio of nucleated to enucleated erythrocytes is evident. The open chromatin structure is shown in the nucleated erythrocyte precursors of mutant embryos in the blood smear preparations (arrow in h).

METHODS. Embryo sections were prepared as described in Fig. 4. Haematoxylin and eosin staining was done for a–d. e–h, Wright–Giemsa stain. For the blood smear, blood was taken from freshly removed embryos at E15.5. The smear was stained with Wright–Giemsa after the blood smear was air-dried.

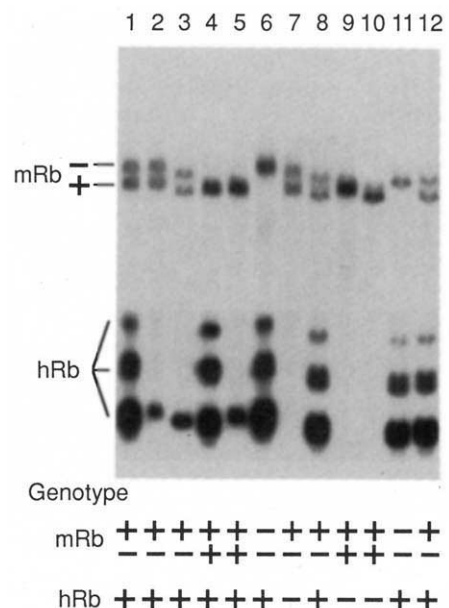


Definition of Rb-deficient embryo abnormalities

Mice heterozygous for the disrupted $Rb-1$ allele were intercrossed to generate homozygotes. No viable $Rb-1^{\Delta 20}/Rb-1^{\Delta 20}$ mice were found when assayed at 3 weeks of age, so embryos from litters at various stages of gestation were genotyped by Southern blot analysis of yolk sac DNA. Development of $Rb-1^{\Delta 20}/Rb-1^{\Delta 20}$ mice is grossly normal up to 10.5 days of gestation (E10.5), during which time there is no apparent selection against homozygous embryos (Table 1). By E11.5, however, some of the mutant embryos show developmental abnormalities, although the majority (71%) still appear normal. By E12.5, many mutant embryos become distorted by swelling, particularly in the region of the fourth ventricle (Fig. 3a, b, e, f). As a result,

FIG. 6 Genetic rescue of the $Rb-1^{\Delta 20}/Rb-1^{\Delta 20}$ mice. Southern blot analysis of tail DNA from the progeny of an $+/Rb-1^{\Delta 20}$; $-/Rb^h \times +/Rb-1^{\Delta 20}$; $-/Rb^h$ cross. The genotype $-/Rb^h$ denotes the hemizygous configuration of the human transgene. The top part of the filter was hybridized with mouse $Rb-1$ probe B; the bottom part was hybridized with a human RB probe. The bottom panel indicates the genotype of each individual mouse. Two $Rb-1^{\Delta 20}/Rb-1^{\Delta 20}$ mice with the human mini RB transgene are shown in lanes 6 and 11.

METHODS. Heterozygous $+/Rb-1^{\Delta 20}$ mice were crossed with mice carrying the human mini RB transgene under its own promoter. F_1 animals transmitting both the $Rb-1^{\Delta 20}$ and Rb^h alleles were intercrossed. Tail DNA was prepared from the F_2 progeny and Southern blot analyses were done as described in Fig. 2.



by E13.5 most (>79%) of the *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos have a distinct hunchback appearance (Fig. 3c, d). In addition, many *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos are pale in colour with poor blood distribution (Fig. 3a, b). The time of onset of the abnormalities seems to vary over a 2–3 day period. Nonetheless, as pregnancy proceeds, all homozygous mutant embryos become runted by E14.0 on a hybrid (C57BL/6×129/J) background. No live homozygotes (determined by heartbeat) have been identified after E16.

Significant defects are found in brain and the blood-forming tissues of the *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos. Most major brain regions develop on schedule, although the overall size of the developing *Rb-1^{Δ20}/Rb-1^{Δ20}* brain is reduced and its cytoarchitecture distorted. Massive amounts of cell death occur throughout the central nervous system (CNS) as early as E11.5 (the earliest age at which sections were examined). The highest concentration of cell death is found in the hindbrain region; telencephalic structures and caudal spinal cord are less affected. The ventricular zone, where neurons and glia are generated, appears normal in most *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos (Fig. 4a, b), but in the intermediate zone, large numbers of dying cells are found (Fig. 4b). Curiously, cell death is less evident among those neurons that successfully complete migration. The peripheral nervous system also forms properly. At E13.5, dorsal root ganglia are present in their proper position and the principal neurons appear to begin normal differentiation (Fig. 4e). Cell death is evident, however, as early as E11.5. By E15.5, the ganglia have withered to nothing but small sheath-like structures (Fig. 4c, d).

A possible cause of the cell loss in *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos is suggested by the discovery of frequent examples of ectopic cell division in the developing CNS. In wild-type embryos, mitotic figures away from the ventricular zone are exceedingly rare. By contrast in *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos, numerous mitotic figures are found in regions normally reserved for neuronal migration or differentiation (inset, Fig. 4f). Further evidence of deregulated proliferation can be found in the developing inferior olive. The cells of the inferior olive migrate tangentially from the rhombic lip along the ventral aspect of the developing hindbrain. In both *+/+* and *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos the olivary stream is visible (although it is wider and less organized in the *Rb-1^{Δ20}/Rb-1^{Δ20}*, Fig. 4g, h). Ectopic cell divisions are common, but cell death is rare in the mutant (Fig. 4i, j), and preliminary cell counts suggest a 30% increase in the number of migrating cells.

The blood-forming tissues of *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos are also defective. During normal development, the major site of haematopoiesis shifts from the yolk sac to the liver (on or before E12). Yolk sac-derived erythrocytes retain their nucleus, whereas liver-generated red blood cells (RBCs) gradually mature and become enucleated. In mutant embryos, the liver is smaller (Fig. 3), but haematopoietic islands are readily identifiable (Fig. 5a, b). Megakaryocytes and myeloid cells are also present. Examination of peripheral blood from E15.5 mutant embryos reveals that most RBCs retain their nucleus (Fig. 5c, d). In contrast, the peripheral blood from wild-type embryos contains mostly enucleated erythrocytes which is characteristic of peripheral RBCs at this age. In Wright–Giemsa strains, hepatocytes were readily identified in wild-type but not in the mutant liver (Fig. 5e, f). Although nucleated erythrocytes are found in both wild-type and mutant embryos, their relative proportions are very different: the less mature forms predominate in the *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos whereas the enucleated types are most common in the wild type. This is further indicated by the open chromatin structure of the nucleated RBCs in the blood smear preparations made from the mutants (Fig. 5g, h). In addition, many of the nucleated RBCs in the mutant embryos have aberrant morphology and staining (Fig. 5).

Normally, the change in the cellular composition of the peripheral blood follows a predictable time course. At E12.5,

nucleated erythrocytes constitute about 75% of the total peripheral blood cells. As development proceeds, this percentage decreases to 50% at E13.5, 25% at E14.5 and 4% at E15.5. In contrast, *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos have virtually no change in the proportion of nucleated erythrocytes in peripheral blood. As in control embryos, about 75% of the total population is nucleated at E12.5. At later stages of development, however, nucleated erythrocytes still represent 65% of the population at E13.5, 70% at E14.5 and 50% at E15.5. Although a slight decrease in the total number of blood cells was noticed in the mutant embryos (Fig. 5c, d), there must be a continuing production of nucleated erythrocytes or a failure of maturation of the nucleated precursor cells to explain the persistence of their relative representation in peripheral blood between E13.5 and E15.5. Taken together the data suggest a combination of abnormal proliferation and differentiation in both the nervous system and the blood of *Rb-1^{Δ20}/Rb-1^{Δ20}* mice.

Genetic rescue of the mutant phenotype

To confirm that the observed mutant phenotype was caused by the mutation in the *Rb-1* gene rather than an alteration elsewhere in the ES cell genome, we introduced a wild-type human *Rb* gene into *Rb-1^{Δ20}/Rb-1^{Δ20}* mice. Transgenic mice carrying a complementary DNA clone of the human *RB* gene (*Rb^h*) under the human *RB* promoter (Y.-J. Bignon and W.-H.L., unpublished results) were crossed to *+/Rb-1^{Δ20}* mice. Because the human transgene is not linked to the endogenous mouse *Rb-1* gene, animals carrying the *Rb^h* as well as the mutated *Rb-1^{Δ20}* allele can be generated by crossing the *Rb^h* transgenic line with the *+/Rb-1^{Δ20}* mice and intercrossing the offspring. Tail DNA of F₂ progeny was analysed for genotype identification. Mice homozygous for the *Rb-1^{Δ20}* allele, but with multiple copies of the *Rb^h* gene are viable (Fig. 6, lanes 6 and 11). Some of these animals have reached 6 months of age with no obvious abnormalities. These results provide evidence that the human *RB* minigene is functional in mice, and that the described phenotype of the mutants is caused by the targeted *Rb-1* disruption.

Discussion

The *Rb-1* gene of mouse embryonic stem cells was modified by homologous recombination using a positive-negative selection strategy³⁹. Disruption of the gene at exon 20 leads to developmental arrest and embryonic lethality in *Rb-1^{Δ20}/Rb-1^{Δ20}* mice. The lethal phenotype of homozygous-deficient mice can be rescued, however, by the presence of a human mini-*RB* transgene. This finding demonstrates both that the human gene (which shares 98% similarity with that of the mouse⁴⁴), can replace the function of the normal mouse *Rb-1* gene and that the homologous recombination event exerts its lethal effect solely through its disabling of the *Rb-1* gene. It is noteworthy that the *Rb-1^{Δ20}* gene lesion removes the T-antigen/E1A binding region of Rb, but leaves most of the N-terminal portion of the protein intact. The implication is that the T/E1A-binding region is required not only for the tumour-suppression activities of Rb, but also for its function in mouse development. Although a complete absence of normal Rb protein was documented in homozygous deficient embryos, several truncated forms are found. These peptides seem to be less stable than wild-type Rb (based on their low abundance in *+/Rb-1^{Δ20}* heterozygotes). Furthermore, their presence does not interfere with the normal function of the wild-type allele because heterozygotes develop normally.

The phenotype of the *Rb-1^{Δ20}/Rb-1^{Δ20}* embryos provides much new information about the role of the Rb protein during development (similar observations are described by Jacks *et al.*⁴³ and Clark *et al.*⁴⁵). Mutant embryos develop in synchrony with their normal litter mates until at least 11.5 days of gestation. Thus, Rb is apparently not essential for the basic processes of pattern formation or cellular proliferation and differentiation, at least up to the early stages of organogenesis. After this,

obvious defects in *Rb-1*^{Δ20}/*Rb-1*^{Δ20} embryos occur in two systems, the CNS and the haematopoietic system. The normal mitotic activity in the ventricular zone of the neural tube suggests that neurogenesis as such does not depend on the *Rb-1* gene. Rather, the massive amounts of cell death that are found in the intermediate zone and maturing neuronal centres suggest that it is the migration and/or differentiation of young, postmitotic neuroblasts that suffers in the absence of a functional *Rb* product. The coexistence of this abnormal cell death with many examples of ectopic mitosis suggests that the phenotype observed in the nervous system derives from the deregulation of cell division.

The deficiencies of haematopoiesis in the *Rb-1*^{Δ20}/*Rb-1*^{Δ20} embryos provides evidence that *Rb* function is not limited to the nervous system. In the embryo, erythropoiesis first occurs in the yolk sac, beginning on E7 and continuing until E12 (ref. 46). After E11, the haematopoietic stem cells migrate to the liver, which then becomes the major site of haematopoiesis. Although hepatic haematopoiesis does occur in the mutant embryos, most erythrocytes are of the less mature, nucleated type. The percentage of the nucleated erythrocytes at E13.5 to E15.5 suggests a deregulation of proliferation of more primitive erythrocytes. Alternatively, failure of the erythrocytes to mature may allow immature precursors to continue to proliferate. Because a reduction in the number of hepatocytes was seen in the mutant mice, further investigation is required to clarify whether the problems are caused by defects in the hepatic microenvironment or in erythroid precursors themselves.

The neurons of the adult brain, with few exceptions, are generated in the ventricular zone during embryogenesis. The presence of numerous mitotic figures outside of this germinative region is noteworthy, but it is unclear why (or whether) ectopic mitosis might lead to the death of neuronal cells. One hypothesis is that the dividing ventricular neuroblasts reach a stage where they switch from a programme of cell division to one of migration and differentiation. The *Rb-1*^{Δ20}/*Rb-1*^{Δ20} cells reach this stage but, in the absence of *Rb*, the postmitotic state is not sustainable. When the cells attempt to go through additional cell divisions, the ongoing progress of differentiation may make division impossible, and thus lead to cell death. If this is correct, then the appearance of the dorsal root ganglia in older *Rb-1*^{Δ20}/*Rb-1*^{Δ20} embryos suggests a corollary to the hypothesis. Nearly all

of the cells of these peripheral nervous system structures die over a relatively short period. It thus seems as if there might be an absolute requirement for *Rb* by these cells during a certain critical period. The suggestion is that different neuronal sub-lineages may have very different requirements for *Rb*. Results from the inferior olive can be seen as supporting this view. Although a 30% increase in the number of migrating olive cells is observed, an increase in cell death is not apparent, thus separating the phenotype of deregulated proliferation from that of abnormal cell death.

The presence of nucleated erythrocytes can also be interpreted as a consequence of abnormal cellular proliferation of a specific lineage due to the absence of *Rb* function. As with the inferior olive neurons, cell death does not seem to be a consequence of the deregulated proliferation of the immature blood cells. Alternatively, *Rb* may be required for maturation of specific erythroid precursors, failure to complete the process may result in the appearance of aberrant erythroid cells. Thus, both the neuronal and haematopoietic phenotypes are consistent with a role of *Rb* in controlling the proliferation/differentiation of a few specific lineages in the embryos.

Curiously, none of the heterozygous mice has developed retinoblastoma. It may be that, as in humans a 'second hit' is required⁴, but because the number of cells in the susceptible target population is at least 10-fold smaller in the tiny eye of a mouse than it is in humans, the probability of tumour formation is too low to lead to observable tumours. In the context of the human condition that led to the isolation of the gene, it is puzzling that the developing neural retina shows no signs of cell loss up to E15.5; both the size and cytoarchitecture of the mutant retina are comparable to the wild type (data not shown). Perhaps the continued proliferation of photoreceptor precursors or the documented phenotype plasticity of the neuronal and glial cell lineages⁴⁷ mitigates the effects of the *Rb*-deficiency. Then too, deregulated cell growth of neuroretinal cells may become evident only at developmental stages beyond E15.5. Experimental approaches that limit the inactivation of the *Rb-1* gene to the retina but allow the generation of viable mice, or the analysis of chimaeric mice made between *Rb-1*^{Δ20}/*Rb-1*^{Δ20} and +/+ cells will provide models to test this hypothesis. □

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- Knudson, A. G. *Birth Defects* **25**, 15–27 (1989).
- Lee, W.-H. In *Tumour Suppressor Genes and Oncogenes* (eds Knudson, A. G., Stanbridge, E. J., Sugimura, T., Terada, M. & Watanabe, S.) 159–170 (Taylor and Francis, London and Bristol, 1990).
- Vogel, F. *Hum. Genet.* **52**, 1–54 (1979).
- Knudson, A. G. *Proc. natn. Acad. Sci. U.S.A.* **68**, 820–823 (1971).
- Cavenee, W. K. *et al. Nature* **305**, 779–784 (1983).
- Dryja, T. P., Rapaport, J. M., Joyce, J. M. & Petersen, R. A. *Proc. natn. Acad. Sci. U.S.A.* **83**, 7391–7394 (1986).
- Friend, S. H. *et al. Nature* **323**, 643–646 (1986).
- Fung, Y. K. T. *et al. Science* **236**, 1657–1661 (1987).
- Lee, W.-H. *et al. Science* **235**, 1394–1399 (1987).
- Abramson, D. H., Ellsworth, R. M., Kitchin, D. & Tung, G. *Ophthalmology* **91**, 1351–1355 (1984).
- Friend, S. H. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 9059–9063 (1987).
- Lee, W.-H. *et al. Nature* **329**, 642–645 (1987).
- Lee, E. Y.-H. P., Huang, S., Shew, J.-Y., Donoso, L. A. & Lee, W.-H. In *Molecular Biology of the Retina: Basic and Clinically Relevant Studies* (eds Faber, D. B. & Chader, G. J.) 221–240 (Wiley-Liss, New York, 1991).
- Bookstein, R., Shew, J.-Y., Chen, P.-L., Scully, P. & Lee, W.-H. *Science* **247**, 712–715 (1990).
- Goodrich, D. W., Chen, Y.-M., Scully, P. & Lee, W.-H. *Cancer Res.* **52**, 1968–1973 (1992).
- Huang, H.-J. S. *et al. Science* **242**, 1563–1566 (1988).
- Sumegi, J., Uzelvoly, E. & Klein, G. *Cell Growth Differ.* **1**, 247–250 (1990).
- Takahashi, R. *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 5257–5261 (1991).
- Lees, J. A., Buchkovich, K. J., Marshak, D. R., Anderson, C. W. & Harlow, E. *EMBO J.* **10**, 4279–4289 (1991).
- Lin, B. T.-Y., Gruenewald, S., Morla, A. O., Lee, W.-H. & Wang, J. Y. J. *EMBO J.* **10**, 857–864 (1991).
- Goodrich, D. W., Duffy, L. A. & Harlow, E. *Cell* **58**, 1097–1105 (1989).
- Chen, P.-L., Scully, P., Shew, J.-Y., Wang, J. Y.-J. & Lee, W.-H. *Cell* **58**, 1193–1198 (1989).
- Ludlow, J. W., Shon, J., Pipas, J. M., Livingston, D. M. & DeCaprio, J. A. *Cell* **60**, 387–396 (1990).
- Mihara, K. *et al. Science* **246**, 1300–1303 (1989).
- Xu, H.-J., Hu, S.-X., Hashimoto, T., Takahashi, R. & Benedict, W. F. *Oncogene* **4**, 807–812 (1989).
- Goodrich, D. W., Wang, N. P., Qian, Y., Lee, E. Y.-H. P. & Lee, W.-H. *Cell* **67**, 293–302 (1991).

- DeCaprio, J. A. *et al. Cell* **54**, 275–283 (1988).
- Dyson, N., Howley, P. M., Munger, K. & Harlow, E. *Science* **243**, 934–937 (1989).
- Whyte, P. *et al. Nature* **334**, 124–129 (1988).
- Rustgi, A. K., Dyson, N. & Bernards, R. *Nature* **352**, 541–544 (1991).
- Bagchi, S., Weinmann, R. & Raychaudhuri, P. *Cell* **65**, 1063–1072 (1991).
- Bandara, L. R. & La Thangue, N. B. *Nature* **351**, 494–497 (1991).
- Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M. & Nevins, J. R. *Cell* **65**, 1053–1061 (1991).
- Chittenden, T., Livingston, D. M. & W. G. Kaelin, J. *Cell* **65**, 1073–1082 (1991).
- Hu, Q., Dyson, N. & Harlow, E. *EMBO J.* **9**, 1147–1155 (1990).
- Huang, S., Wang, N.-P., Tseng, B. Y., Lee, W.-H. & Lee, E. Y.-H. P. *EMBO J.* **9**, 1815–1822 (1990).
- Kaelin, W. G., Jr, Ewen, M. E. & Livingston, D. M. *Molec. cell. Biol.* **10**, 3761–3769 (1990).
- Shew, J.-Y. *et al. Proc. natn. Acad. Sci. U.S.A.* **87**, 6–10 (1990).
- Mansour, S. L., Thomas, K. R. & Capocchi, M. R. *Nature* **336**, 348–352 (1988).
- Adra, C. N., Boer, P. H. & McBurney, M. W. *Gene* **60**, 65–74 (1987).
- Thomas, K. R. & Capocchi, M. R. *Cell* **51**, 503–512 (1987).
- McMahon, A. P. & Bradley, A. *Cell* **62**, 1073–1085 (1990).
- Jacks, T. *et al. Nature* **359**, 295–300 (1992).
- Bernards, R. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 6474–6478 (1989).
- Clarke, A. R. *et al. Nature* **359**, 328–330 (1992).
- Moore, M. A. S. & Metcalf, D. *Br. J. Haemat.* **18**, 279–296 (1970).
- Turner, D. L. & Cepko, C. L. **328**, 131–136 (1988).
- Soriano, P., Montgomery, C., Geske, R. & Bradley, A. *Cell* **64**, 693–702 (1991).
- Bradford, M. M. *Analyt. Biochem.* **72**, 248–254 (1976).
- Huang, S., Lee, W.-H. & Lee, E. Y.-H. P. *Nature* **350**, 160–162 (1991).

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Effects of an *Rb* mutation in the mouse

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The retinoblastoma gene is mutated in several types of human cancer and is the best characterized of the tumour-suppressor genes. A mouse strain has been constructed in which one allele of *Rb* is disrupted. These heterozygous animals are not predisposed to retinoblastoma, but some display pituitary tumours arising from cells in which the wild-type *Rb* allele is absent. Embryos homozygous for the mutation die between days 14 and 15 of gestation, exhibiting neuronal cell death and defective erythropoiesis.

THE retinoblastoma susceptibility gene, *RB*, is the best studied of an expanding list of tumour-suppressor genes which are mutated to an inactive form during the development of a wide range of human tumours¹. Both copies of the *RB* gene are inactivated by somatic mutations during the genesis of sporadic retinoblastoma (a childhood tumour of the eye)²⁻⁴, as well as many osteosarcomas, soft tissue sarcomas, and carcinomas of the breast, lung and bladder⁵⁻¹². In addition, individuals who inherit one defective copy of the *RB* gene have a roughly 90% likelihood of developing retinoblastoma at an early age¹³. This familial form of retinoblastoma is often followed by second malignancies (particularly osteosarcomas) later in life¹⁴. The increased risk of tumour development in individuals who are heterozygous for an *RB* mutation is due to the fact that developing tumour cell clones must suffer only one (instead of the usual two) *RB* mutation during tumour progression¹⁵.

The inactivation of both alleles of *RB* and other tumour suppressor genes during tumorigenesis suggests that they negatively regulate cell growth when functioning normally. The evidence that this is true for *RB* rests largely on functional studies of cloned wild-type *RB* alleles and on biochemical analysis of the gene product, pRb. Introduction of wild-type *RB* into a variety of *RB*⁻ tumour cells *in vitro* reverses tumorigenicity, induces senescence or stops cell proliferation¹⁶⁻¹⁸. The pRb is phosphorylated in a cell-cycle dependent manner¹⁹⁻²¹, and its state of phosphorylation seems to regulate its activity^{22,23}. Also, three DNA tumour virus oncoproteins (adenovirus E1a, simian virus 40 (SV40), large T antigen (T-ag) and human papilloma virus E7) bind to pRb²⁴⁻²⁶ and are thought to inactivate it in virus-transformed cells. Finally, the protein has recently been shown to interact with three cellular transcription factors thought to be involved in cell-cycle regulation: c-Myc, n-Myc and E2F^{27,28}. Taken together, these data have suggested a model in which pRb negatively regulates progression through the cell cycle, perhaps by inhibiting the activity of transcription factors that are required for entry into S phase.

Despite considerable progress in describing the functional biochemistry of pRb, it has been unclear if *RB* plays an important role in normal development or whether it serves solely as a barrier against tumorigenesis postnatally. It is known that in mice *Rb* is expressed early in development, by at least day 11 of gestation²⁹. The almost ubiquitous expression of the *RB* gene in adult tissues^{29,30} is consistent with a general cell-cycle regulatory function. But the relatively small number of tumour types carrying mutations in *RB* suggests that the function of the gene is critical in a more limited range of tissues.

As a means of addressing these and related questions, we have undertaken the construction of a mouse strain in which one allele of the *Rb* gene has been disrupted. This effort has been made possible by the development of methods for gene targeting in mouse embryonic stem (ES) cells³¹. In addition to potentially serving as a model for human familial retinoblastoma (and other human familial cancer syndromes caused by inheritance of a mutant tumour suppressor gene), mice that are heterozygous for an *Rb* mutation can be interbred to create homozygous animals or embryos. An analysis of the homozygous mutant phenotype should help define the function of *RB* in normal cells and provide a basis for understanding the consequences of *RB* mutation in tumorigenesis. We report here on the phenotypes of such *Rb*⁻ heterozygous and homozygous mice.

Rb gene targeting

We have used the positive-negative selection method of Mansour *et al.*³² to disrupt one allele of *Rb* in mouse ES cells. A region of the murine *Rb* gene containing exons 3 and 4 was modified as shown in Fig. 1. A bacterial neomycin gene (*neo*) expression cassette was inserted into the third intron of the gene in place of a roughly 900 nucleotide (nt) fragment of the intron; the direction of transcription of *neo* is opposite to that of *Rb*. In addition, three nucleotide changes were introduced into exon 3 by site-directed mutagenesis, creating two termination codons and a new *Pst*I restriction site within the exon. The herpes simplex virus thymidine kinase (*HSV-tk*) gene was ligated upstream of the *Rb* sequences, in order to allow for counter-selection against ES cells that acquired the targeting vector by random integration of the entire DNA construct³².

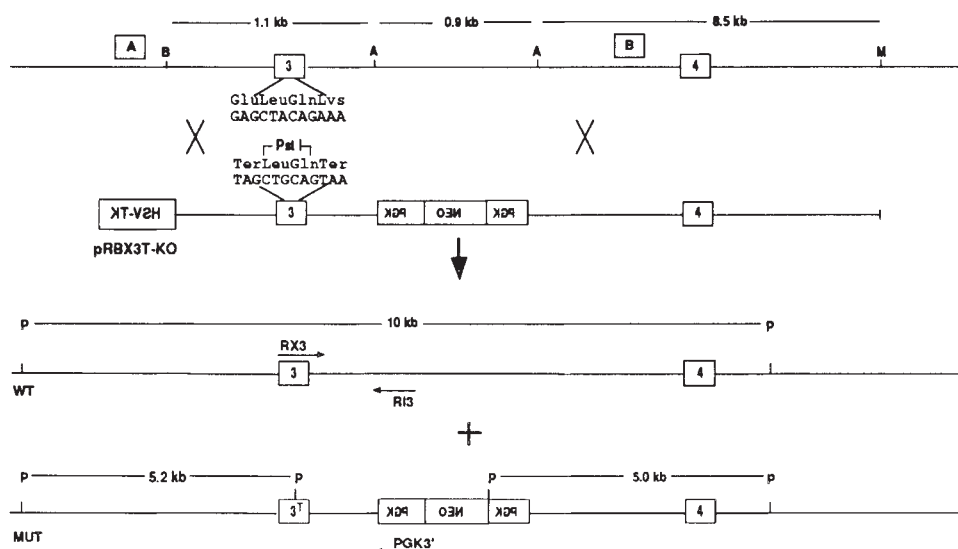
After introduction of the targeting vector into D3 ES cells³³ (derived from strain 129/sv), the resulting *neo*-positive (G418-resistant), *HSV-tk*-negative (gancyclovir-resistant) clones were screened by Southern blotting. Probes located in introns 2 and 3 detect a 10 kilobase (kb) *Pst*I fragment from wild-type DNA and a mutant-specific fragment of about 5 kb (Fig. 1). Of 150 ES clones screened, five had acquired the exogenous *Rb* sequences by homologous recombination and were, therefore, heterozygous for the mutation (not shown); the *HSV-tk* counter-selection resulted in a roughly 10-fold reduction in the number of ES cell colonies. The presence of the point mutations in the third exon was further verified by DNA sequencing of a region of DNA amplified by polymerase chain reaction (PCR) (see Fig. 1; data not shown). Translational termination at the stop codons in exon 3 would result in the synthesis of an N-terminal pRb peptide of $M_r \sim 10K$; full-length mouse pRb has a mass of 105K (ref. 29).

Chimaeric animals were created by injection of heterozygous ES cells into C57BL/6 blastocyst-stage embryos, and contribu-

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FIG. 1 Disruption of *Rb* in ES cells. The *Rb*-targeting vector pRBX3T-KO was constructed by inserting fragments of the mouse *Rb* gene extending from intron 2 to intron 4 (isolated from a strain 129/sv genomic library) into the plasmid pPNT⁴¹. The regions of homology consist of fragments of 1.1 kb (*Bam*HI (B) to Asp 718 (A)) and 8.5 kb (Asp 718 to *Mbo*II (M)). The *pgk-neo* expression cassette is located ~200 nt 3' to *Rb* exon 3 in place of a 900 nt Asp 718 fragment in intron 3. Three base changes were introduced into exon 3, creating two termination codons and a new *Pst*I recognition site. The wild-type and mutant sequences are shown; the altered exon 3 is abbreviated 3'. The HSV-*tk* gene (also under the control of the *pgk* promoter) is located adjacent to the *Rb* intron 2 sequences. The HSV-*tk* and the *pgk-neo* cassette are transcribed in the transcriptional orientation opposite to *Rb*. Homologous recombination between the targeting vector and one chromosomal *Rb* allele produces a mutant *Rb* allele (mut) that can be distinguished from wild-type (wt) *Rb* by Southern blotting of *Pst*I-digested genomic DNA using the probes [A] and [B], which are located on each side of the sites of recombination. The DNA of heterozygous ES cells would contain a wild-type fragment of 10 kb and mutant-specific fragments of ~5 kb. The two alleles can also be distinguished by PCR using primer pairs that specifically amplify wild-type (RX3 and RI3) or mutant sequences (RX3 and PGK3').

METHODS. Electroporation of pRBX3T-KO into D3 ES cells and the subsequent drug selection was done using the conditions described by Tybulewicz *et al.*⁴¹, except that primary mouse embryonic fibroblasts were



used as feeder cells. Genomic DNA was isolated from ES cell clones using the method of Laird *et al.*⁴² and screened by Southern blotting of ~10 µg *Pst*I-digested DNA. Samples were separated in 0.9% agarose gels, transferred to nylon membrane (Hybond-N, Amersham) and hybridized as described⁴³. Blots were initially probed using probe A (which is not contained within the targeting vector), and candidate heterozygous clones rescreened using probe B. The presence of the exon 3 mutations was confirmed by DNA sequencing (Sequenase 2.0, USB) of DNA amplified by PCR (Amplitaq, Perkin-Elmer-Cetus) using primers RX3 and PGK3'. Primer sequences: RX3 (5'-AATTGCGGCGCATCTGCATCTTTATCGC-3'), RI3 (5'-CCCATGTTGCGTCCTAG-3'), PGK3' (5'-GAAGAACGAGATCAGCAG-3').

tion of the injected cells to the germ line of chimaeras was determined by breeding with C57BL/6 animals. Two heterozygous ES clones produced chimaeras which transmitted the *Rb* mutation. Figure 2 shows a representative Southern blot of DNA from several heterozygous and wild-type offspring. Germ line transmission of the *Rb* mutation was also determined by PCR amplification of tail DNA (see Fig. 1; data not shown). We will hereafter refer to this mutant allele of *Rb* as *Rb*^{x3t}, to denote the termination mutations in exon 3.

Tumours in *Rb*^{x3t} heterozygotes

Humans heterozygous for a deleterious mutation in *RB* have a roughly 90% likelihood of developing retinoblastoma by the age of three¹³. In contrast, of more than 100 heterozygous mice that have been followed for up to 11 months of age, not one has developed retinoblastoma. In addition to superficial examination of all heterozygotes, 20 of these animals were screened by indirect ophthalmoscopy, and an additional six eyes were examined by histological sectioning. No signs of either retinoblastoma or precursor lesions (that is, retinomas) were evident.

In addition to their strong predisposition to retinal tumours in early childhood, humans carrying an *RB* mutation have an

increased risk of developing osteosarcoma and other tumours later in life¹⁴. For this reason, we monitored the germ line heterozygotes, as well as the chimaeras made from heterozygous ES cells, for the development of other tumour types. Indeed, we have recently observed the first indication of any cancer predisposition in these animals. Four chimaeras and five germ line heterozygotes were killed after displaying severe wasting between the ages of 8 and 10 months. Autopsy revealed large tumours (~6 mm in diameter) in the area of the pituitary gland in all nine animals. These tumours have been classified histopathologically as adenocarcinoma of the pituitary; a section of one tumour is shown in Fig. 3a. The five pituitary tumours observed in heterozygotes arose in a population of the 20 animals that were at least 9 months old. Southern blot analysis shows an absence of the wild-type allele of *Rb* and retention of the mutant allele in the DNA of eight tumours tested (Fig. 3b; data not shown). This result confirms the role of *Rb* in the genesis of these tumours, and is consistent with the 'two-hit' model of *RB* mutation of the gene first proposed by Knudson¹⁵.

The homozygous phenotype

These heterozygous animals have also been useful for establishing the requirements for *Rb* function during mouse develop-

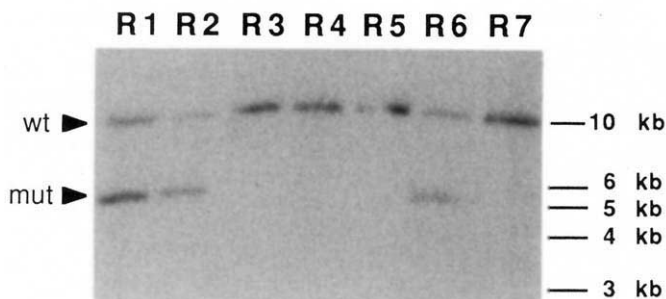


FIG. 2 Germ line transmission of the *Rb*^{x3t} mutation. Southern blotting shows the presence of wild-type (wt) and mutant (mut) *Rb* fragments in the DNA of heterozygous animals (R1, R2, R6). Animals R3, R4, R5 and R7 are homozygous for the wild-type *Rb* allele. The positions of DNA size markers are indicated to the right.

METHODS. C57BL/6 blastocyst-stage embryos were injected with 10–15 mutant D3 ES cells and then transferred to pseudopregnant F₁ (C57BL/6 × DBA/2) females for continued development, essentially as described⁴⁴. Germ line contribution of the injected ES cells was determined by mating chimaeras to C57BL/6 animals; transmission of the D3 (129/sv) genotype produces agouti (rather than black) offspring. Tail DNA was isolated from agouti pups at weaning using the method of Laird *et al.*⁴² and Southern blotting done as described in Fig. 1.

ment. We mated male and female Rb^{x3t} heterozygotes and determined the genotypes of the offspring at 3 weeks of age. If Rb function were dispensable for normal mouse development, we would have expected a 1:2:1 ratio of wild-type, heterozygous and homozygous mutant offspring. If, however, Rb were required for some aspect of embryogenesis, homozygous embryos would not survive to term. The genotypes of the first 64 offspring of such heterozygous crosses revealed 22 wild-type, 42 heterozygous and 0 homozygous animals. This result clearly indicates that Rb is an essential gene in the mouse.

By collecting embryos at progressively earlier times of development, we have been able to delineate the timing of the death of Rb^{x3t} homozygous embryos. Of 70 embryos collected at 12.5 days postcoitum (d.p.c.), 15 were $Rb^{x3t/x3t}$. Because this is within the expected ratio, we can conclude that the Rb^{x3t} mutation does not affect embryonic viability up to day 12.5 of gestation. Furthermore, on a gross morphological level, the $Rb^{x3t/x3t}$ embryos were indistinguishable from their littermates. This viability is not explained by residual expression of Rb , as no pRb is seen in western blots of cell lysates of 12.5 d.p.c. embryos (Fig. 4). We have also shown a lack of pRb expression in $Rb^{x3t/x3t}$ primary embryo fibroblasts using indirect immunofluorescence and immunoprecipitation with a combination of three anti-pRb monoclonal antibodies which recognize different epitopes (data not shown). Because of the lack of high-affinity antibodies recognizing epitopes in the amino-terminal region of pRb, we were unable to assay for the production of the N-terminal 10K fragment of the protein that might be synthesized from the Rb^{x3t} allele.

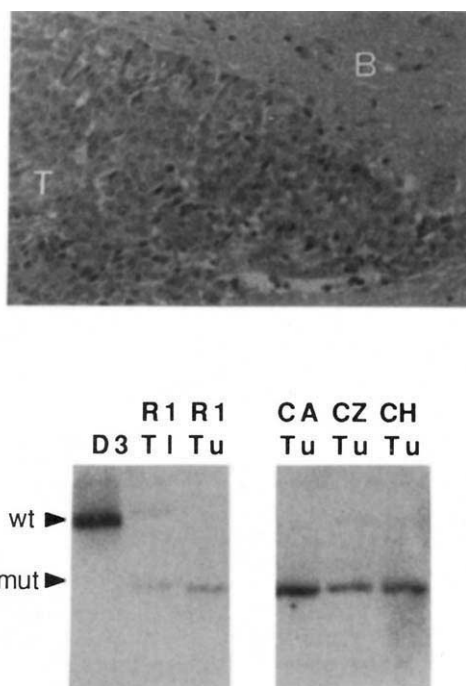


FIG. 3 Pituitary tumours. *a*, Section through a portion of an adenocarcinoma of the pituitary (T) and surrounding brain tissue (B). The cell type of origin of these tumours has not been established. *b*, Southern blot of DNA isolated from pituitary tumours (Tu) from Rb^{x3t} germ line heterozygote R1 and three chimaeras (CA, CZ and CH) made from heterozygous ES cells showing loss of the wild-type (wt) Rb fragment and retention of the mutant (mut) fragment. The R1 tail (TI) and normal D3 ES cell DNA samples serve as heterozygous and homozygous wild-type controls.

METHODS. After surgical removal, pituitary tumours were divided for subsequent histological analysis or DNA isolation. Tissues were fixed in Bouin's fixative, dehydrated in graded solutions of alcohol, embedded in paraffin, sectioned at 6 μ m, and stained with haematoxylin and eosin. Southern blotting was done on *Pst*I-digested genomic DNA as described in Fig. 1.

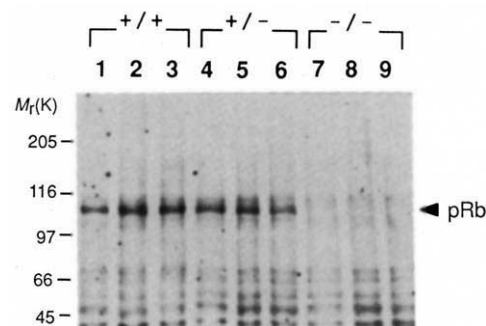


FIG. 4 Lack of full-length pRb in $Rb^{x3t/x3t}$ embryos. Western blotting of brain lysates using an anti-pRb monoclonal antibody reveals high levels of pRb in wild-type (+/+) and heterozygous (+/-) 12.5 d.p.c. embryos and undetectable expression in homozygous mutant (-/-) embryos. Sizes of protein standards are shown at left and the position of migration of pRb is indicated at right.

METHODS. Western blotting was done on lysates of brain tissue isolated from 12.5 d.p.c. embryos using an anti-pRb monoclonal antibody (Pharmingen 14001A) and the Western-Light chemiluminescence detection kit (Tropix). Protein (100 μ g per lane) was separated in a 12% polyacrylamide gel, transferred to nitrocellulose and treated as described⁴⁵. Embryo genotypes were determined using PCR (see Fig. 1) of DNA isolated from the yolk sac.

At day 13.5 of gestation, viable $Rb^{x3t/x3t}$ embryos were also present at the expected frequency (18/53), but at this age most of the homozygotes (14/18) were obviously abnormal. The 13.5 d.p.c. $Rb^{x3t/x3t}$ embryo shown in Fig. 5*a* exemplifies the mutant phenotype: the mutant is paler and has less obvious superficial vasculature than the normal littermate; it also has significant oedema, particularly in the pericardial space, and the liver is slightly reduced in size. The lethality of the Rb^{x3t} mutation was first evident at 14.5 d.p.c., when three of five homozygous embryos were found dead (determined by no heartbeat). All six $Rb^{x3t/x3t}$ embryos recovered at 15.5 d.p.c. were dead.

Inhibition of hepatic erythropoiesis

Largely on the basis of the appearance of the mutant embryos at 13.5 d.p.c. (Fig. 5*a*), we suspected that part or all of the lethal phenotype could be explained by severe anaemia. In fact, a role for Rb in the control of erythroid development was first suggested by earlier work, which demonstrated that at 12.5 days of gestation, the liver was the site of maximal expression of Rb^{29} . At this stage of development, the liver is the major erythropoietic organ³⁴. A block in hepatic erythropoiesis in $Rb^{x3t/x3t}$ embryos could account for the apparent anaemia and might suffice to cause the observed lethality. Furthermore, the survival of these Rb^{x3t} homozygous mutants until day 13 or 14 of gestation suggests that the primitive, yolk sac-derived erythropoiesis, which occurs between days 8 and 11 of gestation³⁴, is not dependent on Rb function.

Examination of the livers and blood of 13.5 d.p.c. mutant embryos indicated substantial disruption of erythropoiesis. Livers of wild-type 13.5 d.p.c. embryos are densely packed with cells (90% of which are in the erythroid lineage) (Fig. 5*b*), whereas the livers of $Rb^{x3t/x3t}$ embryos are lacy in appearance, lacking extensive cellularization. Blood smears from these animals also show evidence of a deficiency of erythrocyte production. We measured the ratio of circulating primitive erythrocytes (nucleated red cells derived in the yolk sac) to the enucleated, definitive erythrocytes born in the liver. Control embryos contain on average 45% enucleated cells at this stage (Fig. 5*d*), whereas $Rb^{x3t/x3t}$ embryos have on average 6.8% enucleated cells (Fig. 5*e*). (These percentages are derived from blood counts of a total of more than 2,000 cells isolated from three control and three mutant embryos.)

The observed inhibition of definitive erythropoiesis in $Rb^{x3t/x3t}$ embryos could be due to a number of factors, including a failure of the haematopoietic stem cells to migrate efficiently to the liver, a defect in the hepatic microenvironment that normally supports erythropoiesis, or an intrinsic inability of Rb^- erythroid progenitor cells to achieve end-stage differentiation. We have begun to examine the Rb requirement in this system by determining the *in vitro* differentiation capacity of erythroid precursors isolated from mutant and control embryos. Single-cell suspensions made from the livers of 12.5 d.p.c. $Rb^{x3t/x3t}$ and control embryos were plated *in vitro* under conditions optimized for erythroid differentiation³⁵. Plates were seeded with equal numbers of cells, although we consistently observed two- to fivefold fewer cells in the livers of homozygous mutant embryos. Erythroid colonies were then examined on each of the first 7 days after plating.

We observed comparable numbers of small haemoglobinized (CFU-E) colonies from mutants and controls three days after plating. Thus, the failure of $Rb^{x3t/x3t}$ embryos to produce definitive erythrocytes efficiently, is not a consequence of the absence of the relevant precursor cells in the liver. But the CFU-E colonies derived from Rb homozygotes differed qualitatively from controls, being slightly paler in colour (Fig. 6a, b). Also, control CFU-E colonies were visible up to 5 days in culture, whereas the mutant CFU-E colonies were no longer apparent at this time. Large haemoglobinized colonies from $Rb^{x3t/x3t}$ embryos were also paler than controls (not shown), and at day 4 were three times more numerous in mutant samples than in control samples.

A number of colonies from mutant and control platings were picked and stained with Wright-Giemsa. Control CFU-E colonies contain a significant fraction of enucleated erythrocytes (Fig. 6c), whereas the mutant CFU-E colonies have very few if any of these mature red cells (Fig. 6d). Instead, the mutant colonies contain increased number of a small, very densely staining cell type that most resembles a late normoblast. Similarly, large erythroid colonies from control embryos consist of about 45% enucleated erythrocytes (Fig. 6e); mutant colonies have fewer than 5% enucleated cells (Fig. 6f). Thus, $Rb^{x3t/x3t}$ erythroid precursors seem to be intrinsically defective in reaching end-stage differentiation. This result is consistent with the

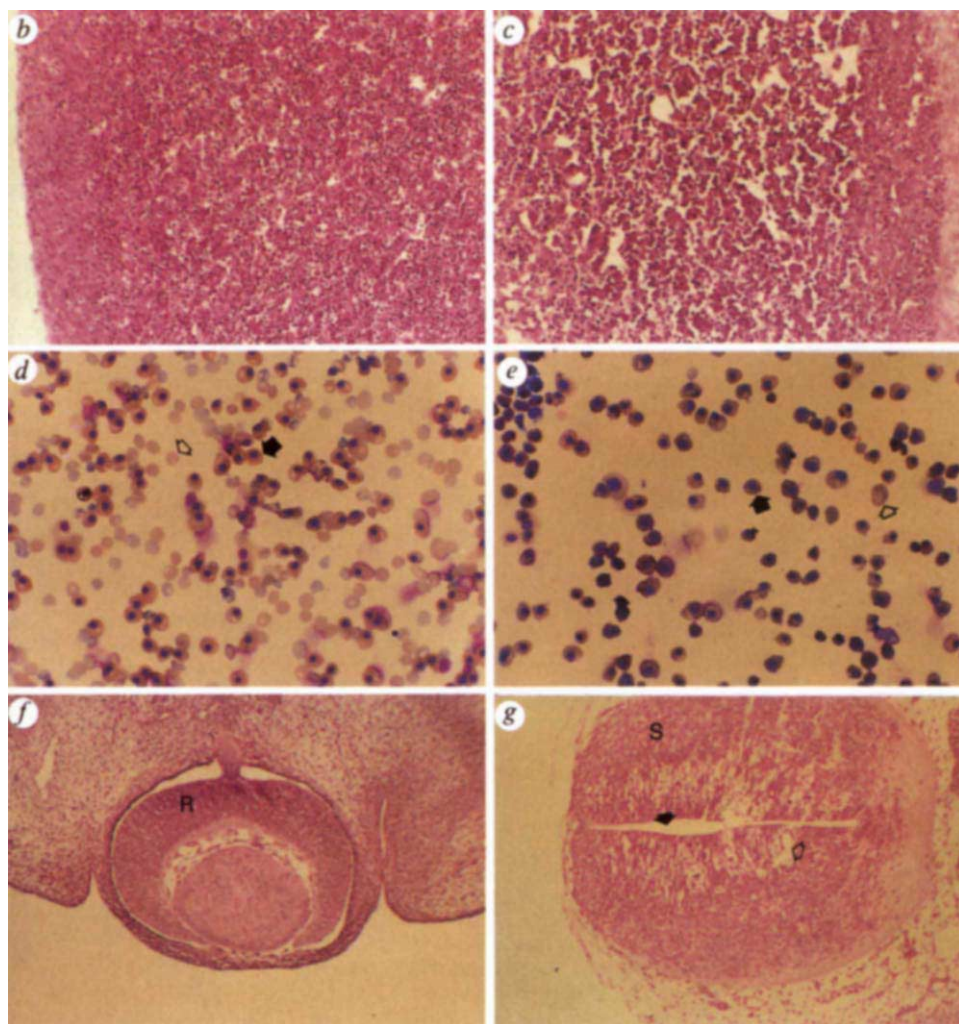


FIG. 5 a, A 13.5 d.p.c. $Rb^{x3t/x3t}$ embryo (left) with a control wild-type littermate. Histology of mutant and control 13.5 d.p.c. embryos. b, Cross-section through wild-type liver. c, Cross-section through mutant liver. Note areas of acellularity. d, Wild-type peripheral blood. e, Mutant peripheral blood. Enucleated, definitive erythrocytes (open arrows) and nucleated, primitive erythrocytes (filled arrows) are indicated. f, Cross-section of eye from a $Rb^{x3t/x3t}$ embryo showing normal retinal (R) development. g, Cross-section through spinal cord (S) of a mutant embryo showing areas of increased cell death (open arrow). The neural canal (filled arrow) is also indicated.

METHODS. Embryos were prepared for histology as described in Fig. 3 and serially cross-sectioned at 6 μ m. Sections were stained with haematoxylin-eosin. Embryonic peripheral blood was obtained from the umbilical vein and artery and stained with Wright-Giemsa.

inhibition of definitive erythropoiesis observed *in vivo*. Our conclusion from this analysis is that $Rb^{x3t/x3t}$ embryos are unable to produce sufficient numbers of mature erythrocytes beginning at about day 13 of gestation. This condition would be expected to lead to hypoxia and the eventual death of the embryo.

Neuronal cell death

We have histologically analysed several Rb^{x3t} homozygous mutants for evidence of other effects of the lack of *Rb* expression during development. With the exception of damage to the dermis and underlying mesenchyme from oedema, most non-haematopoietic tissues appear normal until the death of the $Rb^{x3t/x3t}$ embryos at about 14.5 d.p.c. But serial cross-sections from day 12.5 and 13.5 embryos have shown fairly widespread neuronal cell death in mutants compared to controls. Evidence of increased cell death was observed in the spinal cord (Fig. 5g), as well as in the dorsal root ganglia and portions of the hindbrain. The cell death does not seem to be a secondary effect of hypoxia induced by anaemia, because it is evident at 12.5 d.p.c., before the manifestation of the erythropoietic defect. In addition to necrosis there is a marked increase in number of mitotic figures in and away from the ventricular zone of the spinal cord and the hindbrain of the mutant embryos⁴⁶. Finally, we paid particular attention to the retinas of Rb^{x3t} homozygous

mutants for any evidence of neoplastic transformation. As shown in Fig. 5f, however, up to day 13.5 of gestation, the absence of *Rb* function does not seem to affect development of the eye.

Discussion

Familial retinoblastoma is a highly penetrant disease in humans. About 90% of carriers develop retinal tumours by the age of three¹³; these individuals have on average six tumour foci, often affecting both eyes. In stark contrast, the heterozygous mice reported here, which carry an analogous mutation in *Rb*, are not predisposed to retinoblastoma. We have considered the possibility that this species difference is due to the relatively small number of target cells in the mouse retina in which inactivation of the wild-type allele of *Rb* would lead to transformation. But the apparent normality of the retinas of 13.5 d.p.c. $Rb^{x3t/x3t}$ embryos argues that even if Rb^- retinoblasts were produced in heterozygous mice, they would not be transformed.

Retinoblastoma has, nonetheless, been observed in mice³⁶. A transgenic strain expressing SV40 T-antigen in the retina develops this tumour at a high frequency. In light of our results, we believe that transformation in this system involves functions of T-antigen beyond its effects on pRb²⁵, including, for example, sequestration of the p53 protein^{37,38}. Therefore, the failure to observe retinoblastoma in Rb^{x3t} -heterozygous mice may be explained by a requirement for mutations in additional genes. Spontaneous retinoblastoma has not been observed in any species besides humans, perhaps suggesting that human retinoblasts are uniquely sensitive to the simple loss of *RB* function.

Although mice that are heterozygous for an *Rb* mutation do not develop retinoblastoma, they are predisposed to malignancy. All nine pituitary tumours described here arose from cells that were heterozygous for the Rb^{x3t} mutation and eight were shown to have lost the wild-type allele of the gene. This is a strong indication of *Rb* involvement in pituitary tumorigenesis in these cases and is the first example of *Rb* mutation in any primary mouse tumour. It is not known whether *RB* is mutated in human pituitary tumours.

We have also established that *Rb* is an essential gene in the mouse. Homozygous mutant embryos die between days 13.5 and 15.5 of gestation, apparently from a failure to produce definitive erythrocytes efficiently in the liver. Given the known association of pRb with the transcription factors c-Myc, N-Myc and E2F^{27,28}, this developmental defect might be explained by the ability of pRb to associate with and potentiate the activity of transcription factors that are essential for erythroid-specific gene expression. The haematopoietic phenotype of embryos deficient for *c-myc* is very similar to that described here³⁹, suggesting a possible functional connection between c-Myb and pRb.

An alternative model, more in keeping with the putative role of pRb as a cell-cycle regulator, would state that pRb acts to cause a cessation of cell division which is a prerequisite to terminal differentiation. Accordingly, the absence of pRb may trap precursor cells of particular lineages in a proliferative compartment and preclude entry into end-stage differentiation. Indeed, there is evidence of increased mitotic activity in the developing nervous system of the $Rb^{x3t/x3t}$ mutant embryos, although it is not known whether there is also increased proliferation of haematopoietic precursors in these animals. To explain the observed reduction in cell number in these lineages, it is necessary to argue that the inability to terminally differentiate ultimately leads to cell death. It is also possible that the observed increase in mitotic activity in the nervous system occurs in response to increased cellular turnover.

We have been struck by the apparent dispensability of *Rb* function for much of mouse development. It would seem that *Rb* does not play a critical role in regulating cell division or differentiation up to day 13–14 of gestation. Alternatively, in the absence of *Rb* expression, an *Rb*-related gene, such as *p107* (ref. 40), might provide an analogous function in most tissues.

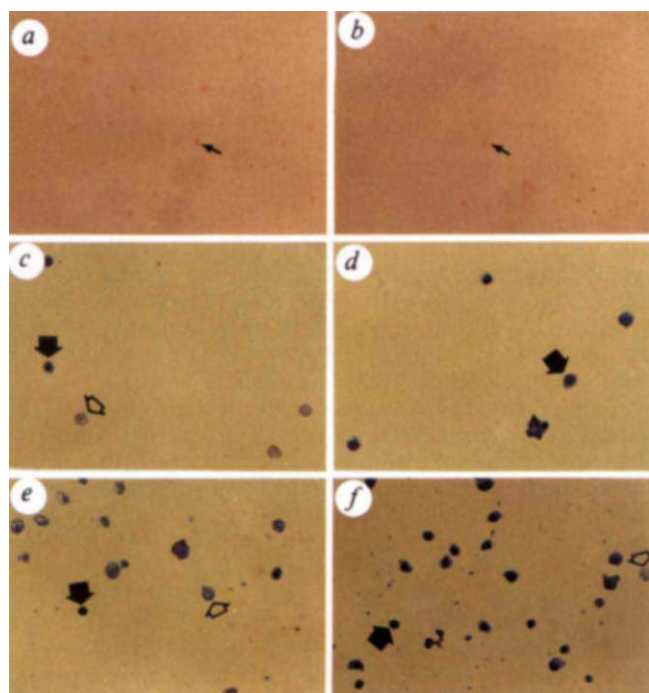


FIG. 6 *In vitro* differentiation of control and mutant erythroid precursors. *a*, Wild-type CFU-E colonies. *b*, Mutant CFU-E colonies. Arrows point to single CFU-E colonies. These mutant CFU-E colonies appear slightly paler in colour than controls. *c*, Enucleated erythrocytes (open arrows) and nucleated cells (filled arrows) derived from a wild-type CFU-E colony. *d*, Nucleated cells (filled arrow) derived from mutant CFU-E colony. *e*, Isolated cells of a wild-type large erythroid colony showing enucleated erythrocytes (open arrows) and nucleated cells (filled arrows). *f*, Isolated cells of an $Rb^{x3t/x3t}$ large erythroid colony composed predominantly of nucleated cells (closed arrow) and a few enucleated erythrocytes (open arrow).

METHODS. Methylcellulose (Terry Fox Laboratories) cultures were set up as described³⁵ using single-cell suspensions from livers of $Rb^{x3t/x3t}$ and control 12.5 d.p.c. embryos. Cells were seeded at 10^5 per 3.5 cm dish in the presence of erythropoietin (2 units ml^{-1} ; Amgen) and 1% pokeweed mitogen-stimulated spleen cell conditioned medium (Terry Fox Laboratories). Colonies were examined on each of the first 7 days after plating. Cells isolated from CFU-E and large erythroid colonies were stained with Wright-Giemsa.

The *Rb*-homozygous embryos offer advantages beyond the insights into regulation of erythropoietic and neuronal lineages described here. Study of the role of *Rb* inactivation in tumour progression has been confounded by the multitude of other genetic lesions that are invariably present in tumour-cell

genomes. The *Rb*^{x3t/x3t} homozygous embryos now provide a source of a variety of cell types that, although lacking *Rb* function, would seem to be genetically otherwise intact. Such cells should prove invaluable in dissecting out the unique role that this gene plays in regulation of cell proliferation. □

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- Weinberg, R. A. *Science* **254**, 1138–1146 (1991).
- Lee, W. J. *et al. Science* **235**, 1394–1399 (1987).
- Friend, S. H. *et al. Nature* **323**, 643–646 (1986).
- Fung, Y. K. *et al. Science* **236**, 1657–1661 (1987).
- Bookstein, R. *et al. Proc. natn. Acad. Sci. U.S.A.* **87**, 7762–7766 (1990).
- Friend, S. H. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 9059–9063 (1987).
- Harbour, J. W. *et al. Science* **241**, 353–356 (1988).
- Hensel, C. H. *et al. Cancer Res.* **50**, 3067–3072 (1990).
- Lee, E. Y.-H. *et al. Science* **241**, 218–221 (1988).
- T'Ang, A., Varley, J. M., Chakraborty, S., Murphree, A. L. & Fung, Y.-K. T. *Science* **241**, 1797–1800 (1988).
- Varley, J. M. *et al. Oncogene* **4**, 725–729 (1989).
- Yokota, J. *et al. Oncogene* **3**, 471–475 (1988).
- Matsunaga, E. *Humangenetik* **56**, 127–128 (1980).
- Abramson, D. H., Ellsworth, R. M., Kitchin, F. D. & Tung, G. *Ophthalmology* **91**, 1351–1355 (1984).
- Knudson, A. G. *Proc. natn. Acad. Sci. U.S.A.* **68**, 820–823 (1971).
- Bookstein, R., Shwe, K. J. Y., Chen, P. L., Scully, P. & Lee, W. H. *Science* **247**, 712–715 (1990).
- Huang, H. J. *et al. Science* **242**, 1563–1566 (1988).
- Qin, X., Chittenden, T., Livingston, D. M. & Kaelin, W. G. *Genes Dev.* **6**, 953–964 (1992).
- Buchkovich, K., Duffy, L. A. & Harlow, E. *Cell* **46**, 447–456 (1989).
- DeCaprio, J. A. *et al. Cell* **58**, 1085–1095 (1989).
- Chen, P.-L., Scully, P., Shew, J.-Y., Wang, J. Y. J. & Lee, W.-H. *Cell* **58**, 1193–1198 (1989).
- Mittnacht, S. & Weinberg, R. A. *Cell* **65**, 381–393 (1991).
- Ludlow, J. W. *et al. Cell* **56**, 57–65 (1989).
- Whyte, P. *et al. Nature* **334**, 124–129 (1988).
- DeCaprio, J. A. *et al. Cell* **54**, 275–283 (1988).
- Dyson, N., Howley, P. M., Munger, K. & Harlow, E. *Science* **243**, 934–937 (1989).
- Rustgi, A. K., Dyson, N. & Bernards, R. *Nature* **352**, 541–544 (1991).

- Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M. & Nevins, J. R. *Cell* **65**, 1053–1061 (1991).
- Bernards, R. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 6474–6478 (1989).
- Lee, W.-H. *et al. Nature* **329**, 642–645 (1987).
- Capecchi, M. *Science* **244**, 1288–1292 (1989).
- Mansour, S. L., Thomas, K. R. & Capecchi, M. R. *Nature* **336**, 348–352 (1988).
- Gossler, A., Doetschman, T., Korn, R., Serfling, E. & Kemler, R. *Proc. natn. Acad. Sci. U.S.A.* **83**, 9065–9069 (1986).
- Russell, E. S. *Adv. Genet.* **20**, 357–459 (1979).
- Wong, P. M. C., Chung, S. W., Chui, D. H. K. & Eaves, C. J. *Proc. natn. Acad. Sci. U.S.A.* **83**, 3851–3854 (1986).
- Windle, J. J. *et al. Nature* **343**, 665–668 (1990).
- Linzer, D. I. J. & Levine, A. J. *Cell* **17**, 43–52 (1979).
- Lane, D. P. & Crawford, L. V. *Nature* **278**, 261–263 (1979).
- Mucenski, M. L. *et al. Cell* **65**, 677–689 (1991).
- Ewen, M. E., King, Y., Lawrence, J. B. & Livingston, D. M. *Cell* **66**, 1155–1164 (1991).
- Tybulewicz, V. L. J., Crawford, C. E., Jackson, P. K., Bronson, R. T. & Mulligan, R. C. *Cell* **65**, 1153–1163 (1991).
- Laird, P. W. *et al. Nucleic Acids Res.* **19**, 4293 (1991).
- Church, G. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1991–1995 (1984).
- Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: a Practical Approach* (ed. Robertson, E. J.) 113–152 (IRL, Oxford, 1987).
- Bronstein, I., Voyta, J. C., Murphy, O. J., Bresnick, L. & Kricka, L. J. *Biotechniques* **12**, 748–753 (1992).
- Lee, E. Y.-H. *et al. Nature* **359**, 288–294 (1992).

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LETTERS TO NATURE

X-ray detection of the eclipsing millisecond pulsar PSR1957+20

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IN the binary millisecond pulsar system PSR1957+20 (ref. 1), a wind from the pulsar is ablating a low-mass (0.02 solar mass) companion and also inflating a local nebula² confined by the ram pressure of the interstellar medium. We have detected X-ray emission from this system, using the Rosat satellite. X-ray emission is expected from the pulsar magnetosphere and the two shocks of the pulsar wind, one at the companion and the other inside the nebula. Our observations show that less than 20% of the pulsar's spin-down luminosity can be carried away by electrons and positrons with Lorentz factor $\gamma \approx 10^5$, and less than 5% by electrons and positrons with $\gamma \approx 10^8$. Neither of these fluxes can provide the penetrating flux required to heat the companion's photosphere. These observations and those in the accompanying paper by Fruchter *et al.*³ represent the first direct diagnostics of the relativistic wind from a weakly magnetized pulsar, and suggest that the wind differs substantially from that of the more highly magnetized Crab pulsar.

The composition of the relativistic wind, which removes energy at a rate $L_{\text{psr}} = I\Omega\dot{\Omega}$ as the pulsar spins down, is poorly known. Many models^{4–6} of pulsar magnetospheres suggest that this wind is primarily electromagnetic radiation, with little relativistic particle content. In contrast, the Crab pulsar reveals nebular radiation that is most easily understood⁷ as being produced when a wind dominated by ultrarelativistic electrons and

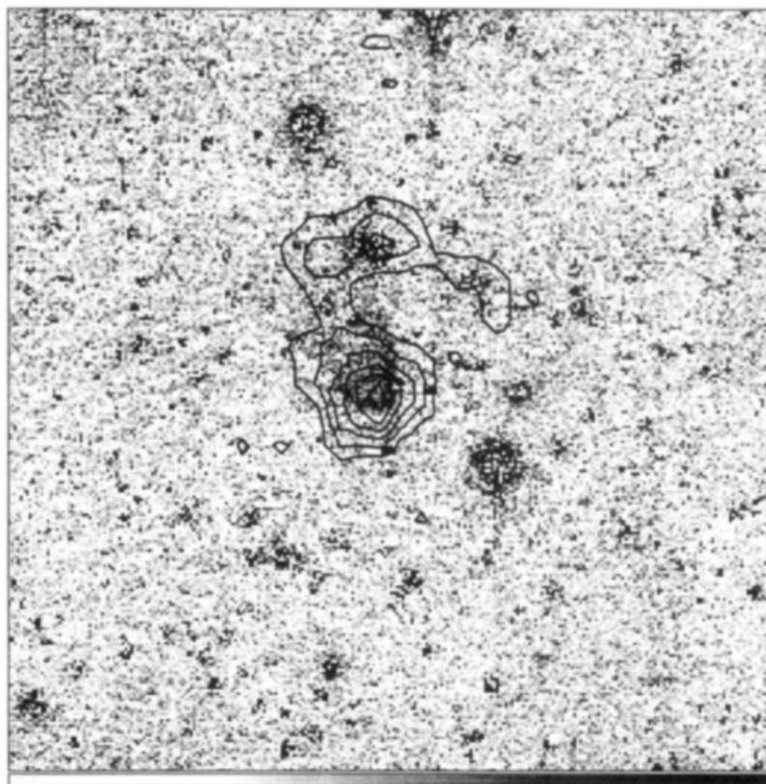
positrons (hereafter 'electrons' should be taken to mean e^+ and e^-) passes through a reverse shock. It has been argued⁸ that these points are reconciled if the pulsar wind becomes increasingly particle-dominated as it travels outwards, with magnetic reconnection increasing the mean particle Lorentz factors γ .

The eclipsing pulsar system PSR1957+20 (refs 1, 9), consisting of a 1.61-ms pulsar and a 0.02 solar mass ($M_\odot$) dwarf companion separated by $a = 1.7 \times 10^{11}$ cm, and surrounded by a H α bow shock nebula², offers new opportunities to probe the composition of a pulsar wind. Energetic electrons from the pulsar may produce X-rays, either in the pulsar magnetosphere or through interactions with the atmosphere of the companion or with the interstellar medium (ISM). We were interested in whether the wind from a weakly magnetized pulsar such as PSR1957+20 (surface magnetic field $B_{\text{surf}} \approx 2 \times 10^8$ G) differs from the wind from a highly magnetized pulsar such as the Crab ($B_{\text{surf}} \approx 4 \times 10^{12}$ G). We therefore observed PSR1957+20 with the Position Sensitive Proportional Counter¹⁰ (PSPC) aboard the X-ray satellite Rosat.

X-ray emission is seen close to the pulsar timing position⁹ (Fig. 1) with $\Delta\alpha = -0.42$ s, $\Delta\delta = 7$ arcsec (differences are in the sense of pulsar timing position minus that given by PSPC). These differences are within the expected (systematic) Rosat position error of 15 arcsec (90% probability error radius). A point X-ray source located 452 arcsec to the north of the pulsar, at $\alpha(2000) = 19$ h 59 min 36.33 s and $\delta(2000) = 20^\circ 40' 42.6''$ (outside the field of view of Fig. 1) appears to coincide with a visual 11.3 mag star in the Hubble Space Telescope (HST) guide-star catalogue¹¹. Many stars have associated X-ray emission, mainly due to chromospheric emission. Assuming that the association is real allows us to improve the absolute astrometry of the PSPC image. The new location of the X-ray peak results in $\Delta\alpha = -0.22$ s, $\Delta\delta = -6$ arcsec. Within the systematic error of the PSPC (2 arcsec) and the uncertainty due to Poisson statistics (3.5 arcsec), the X-ray peak is consistent with the pulsar timing position.

To first order, the X-ray emission contours are consistent with those of a point source. (The emission 72 arcsec north of the pulsar can be attributed to a star of visual magnitude $V = 10.9$,

FIG. 1 Contour map of the X-ray emission in the hard band (0.5–2.0 keV) of the region around PSR1957 + 20. The X-ray observations were made during the period 21–23 October 1991, and the total on-source integration time was 14.5×10^3 s. The underlying grey scale is an image² obtained at the Palomar Observatory using a 15-Å filter centred on H α ; the continuum has been subtracted. The image is a square with a 5 arcmin side.



which is seen in Fig. 1 of ref. 12 and is also present in the HST guide-star catalogue¹¹). The estimated visual extinction, $A_V \approx 1$ –2 (ref. 13), corresponds to a mean hydrogen column density of $N_H \approx 4.5 \times 10^{21} \text{ cm}^{-2}$. Restricting the analysis to the hard band (0.5–2.0 keV), a reasonable choice given the probable high N_H , led to little loss of source photons and a decrease in background rate by a factor of 3. Within the 25 arcsec radius of the peak we detected a total of 32 photons. The mean background count over many such apertures elsewhere in the image was found to be 3 ± 0.13 (1σ). Thus the background-corrected signal count rate over this aperture is $2 \times 10^{-3} \text{ s}^{-1}$. Assuming a Crab-like spectrum, we derive an X-ray flux $F_X(0.5\text{--}2.0 \text{ keV}) \approx 7 \times 10^{-14} \text{ erg cm}^{-2} \text{ s}^{-1}$. The inferred X-ray luminosity is $L_X = 4\pi D^2 F_X \approx (8 \pm 1.3) \times 10^{30} D_{\text{kpc}}^2 \text{ erg s}^{-1}$; here the distance is D_{kpc} (in kiloparsecs) and the uncertainty reflects 1σ errors due to Poisson statistics.

One possible interpretation of F_X is X-ray emission from the pulsar magnetosphere. This would not be affected by the tenuous plasma that causes the radio eclipses⁹, so such emission would not show orbital modulation. The photon arrival times were mapped to a value between 0 and the total integration time. A Kolmogorov–Smirnov test¹⁴ of these remapped arrival times resulted in $D = 0.19$. The probability of obtaining $D > 0.19$ is 0.17 and hence we conclude that the arrival times could be consistent with a time-invariant intensity source. This test is not particularly sensitive to modulations when the data are, like ours, spread over many orbital periods. Remapping the arrival times to orbital phase does not reveal any significant modulation (Fig. 2). The pulsar's magnetospheric emission, assuming no beaming, is $\leq 5 \times 10^{-5} L_{\text{psr}}$, well below the 10^{-3} fraction of emission from the Crab pulsar in the same band¹⁵; here $L_{\text{psr}} \approx 1.6 \times 10^{35} \text{ erg s}^{-1}$ is the pulsar spin-down luminosity.

X-rays produced near the companion will also be unresolvable. Relativistic electrons will emit synchrotron radiation in the shock-heated and decelerated pulsar wind downstream of the bow shock in the pulsar wind¹⁶ surrounding the companion star. For electrons with $\gamma \approx 10^5$, peak photon energies $\epsilon \approx 0.7(\gamma/10^5)^2(B/14 \text{ G}) \text{ keV}$ will be in the Rosat band. The strength of the emission is determined by the number of shocked electrons

and the ratio of the radiative cooling time to the time for the electrons to cool by expansion behind the obstacle. Such emission would be beamed in a forward cone (because the shocked fluid is accelerated by the pressure gradient as it flows around the eclipse region). Relativistic beaming would tend to give maximum flux just before and after the pulsar eclipse phase ($\phi = 0.25$). Thus any such emission should be distinguished by its modulation at the orbital period P_b .

Near the companion, a fraction $f_{\text{min}} \approx (R_E/2\alpha)^2 = 2 \times 10^{-2}$ of L_{psr} is incident on the eclipse region, where $R_E \approx 5 \times 10^{10} \text{ cm}$ is the radius of radio-beam eclipse. This fraction might be larger if the orbit were inclined or the pulsar wind were anisotropic. The fractions of L_{psr} carried by relativistic particles and by the magnetic field are not known. We take σ_p to be the ratio of Poynting flux to particle energy flux⁷, and consider first magnetically dominated or equipartition winds ($\sigma_p \geq 1$), for which the post-shock magnetic field is $B \approx 14 \text{ G}$. The fraction f_5 of the energy flux in relativistic electrons with post-shock Lorentz factors of $\gamma \approx 10^5$ will be able to radiate in the Rosat band. Corresponding pre-shock Lorentz factors are $2.5 \times 10^5 \sigma_p^{1/2}$. These electrons cool over a time $t_{\text{cool}} = 22(\gamma/10^5)^{-1}(B/14 \text{ G})^{-2} \sigma_p^{3/2} \text{ s}$ in the pulsar rest frame, or about $6\sigma_p^{3/2}$ times longer than the flow time $t_{\text{flow}} = 2R_E/c$ around the eclipse region. A fraction $f_{\text{rad}} = t_{\text{flow}}/t_{\text{cool}} \approx 0.15\sigma_p^{-3/2}$ of the electron energy will be radiated. The expected X-ray luminosity near times of pulsar eclipse is thus a few times $f_{\text{min}} f_5 f_{\text{rad}} I \Omega \Omega$, so the flux at the Earth would be

$$F_{P_b} \equiv f_{P_b} F_X \approx 4.1 \times 10^{-12} f_5 I_{45}^2 D_{\text{kpc}}^{-2} \sigma_p^{-3/2} \text{ erg cm}^{-2} \text{ s}^{-1} \quad (1)$$

The apparent modulation in Fig. 2 is suggestive of $f_{P_b} \approx 1$, but given the photon statistics, is unfortunately not significant. Thus we can only limit $f_5 < 2 \times 10^{-2} I_{45}^2 D_{\text{kpc}}^2 \sigma_p^{3/2}$, up to a maximum of $f_5 \approx 0.2 \approx \sigma_p^{-1}$. This is already in striking contrast to $f_5 \approx 1$ for the Crab pulsar⁸. A similar calculation for a particle-dominated wind ($\sigma_p \leq 0.1$) gives $f_5 < 2 \times 10^{-2} I_{45}^{-7/4} D_{\text{kpc}}^2 (\sigma_p/0.1)^{-3/4}$. If $f_{P_b} \approx 1$, then $6 \times 10^{-4} < \sigma_p < 4$.

On closer inspection of Fig. 1, some asymmetry in the X-ray emission is apparent, with a tail pointing along the axis of symmetry of the nebula. Comparing the contour map of this

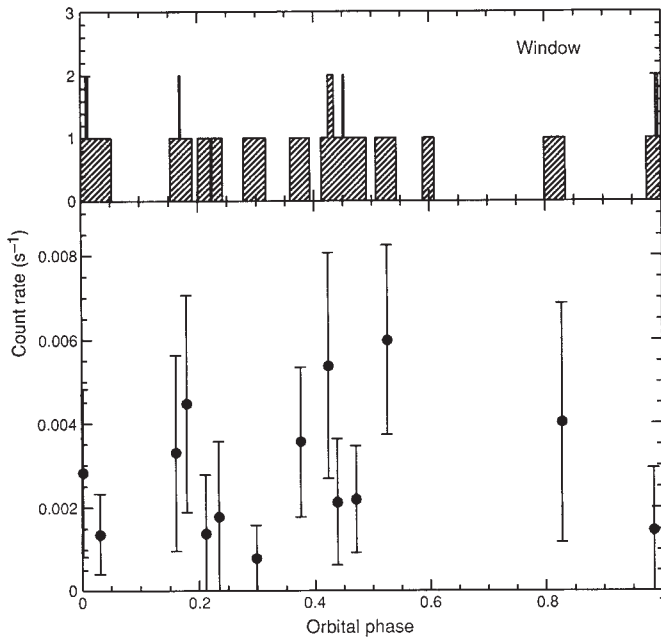


FIG. 2 X-ray count rate from the unresolved source coincident with PSR1957+20, against the pulsar's orbital phase. Counts are folded with the pulsar's 33,002-s orbital period⁹. The upper panel shows the orbital coverage of the observations. Roughly 42% of the orbit is sampled. The lower panel shows the X-ray count rates derived from photons received during these observing periods in the hard band (0.5–2.0 keV) and within a 30-arcsec aperture centred on PSR1957+20. A background rate of $4 \times 10^{-4} \text{ s}^{-1}$, as measured off-source, is expected within this aperture. If correlation with orbital phase is neglected, the distribution of counts in each observing period is consistent with Poisson statistics and a mean rate above background of $2 \times 10^{-3} \text{ s}^{-1}$. Taking orbital phase into account, there are suggestive but not significant increases in flux before (at phase $\phi \approx 0.17$) and after (at phases $\phi \approx 0.4$ – 0.5) the phase coinciding with pulsar radio eclipse ($\phi = 0.25$). Modulation of this sort is predicted because of relativistic beaming in the flow as it is channelled around the eclipse region. Further observations will be required to confirm the modulation.

emission with that of a point source with similar photon statistics shows that at most the observed emission deviates from a point source at contour levels $\leq 25\%$ of the peak value. Given the low photon statistics and the angular resolution of the PSPC (25 arcsec full width at half maximum, on axis) we conclude that the nebular flux $f_{\text{Neb}} F_X$ is limited to $f_{\text{Neb}} < 0.5$. Similar limits are obtained³ from a higher-spatial-resolution X-ray image of this field.

This upper limit on f_{Neb} allows us to draw additional interesting constraints. The H α bow-shock nebula² results as the entire system moves at high speed through dense ISM (number density $n_H \geq 1 \text{ cm}^{-3}$) and the pulsar-wind momentum flux opposes the ISM ram pressure. The observed proper motion⁹ implies a maximum ISM shock velocity of $140 D_{\text{kpc}} (\sin \alpha)^{-1} \text{ km s}^{-1}$, where α is the angle between the pulsar's velocity and our line of sight. This yields post-shock ISM gas temperatures too low ($kT \approx 50 \text{ eV} (\sin \alpha)^{-2}$) to account for the observed X-rays.

Inside the H α shell, nearly all of the pulsar wind passes through a reverse shock, downstream of which electrons emit synchrotron radiation as in the Crab nebula. The most intense synchrotron X-ray emission might be expected at the apex of the bow shock, located $d_s \approx 0.02 D_{\text{kpc}} (\sin \alpha)^{-1} \text{ pc}$ from the pulsar. At this distance, assuming an equipartition or particle-dominated pulsar wind, the field is $B = 14 (\sigma_{\text{neb}}/1 + \sigma_{\text{neb}})^{1/2} a/d_s G \approx 4 \times 10^{-5} (\sigma_{\text{neb}}/1 + \sigma_{\text{neb}})^{1/2} G$, and electrons radiating effectively in the 1-keV band require $\gamma \approx 7 \times 10^7 \sigma_{\text{neb}}^{-1/4}$. Such Lorentz factors are similar to those predicted by extrapolations¹⁷ of theories of the Crab's pair wind, but may also arise from particle acceleration¹⁸ in the reverse shock and need not be representative of the pre-shock wind.

After encountering the reverse shock, the wind will be deflected as a mildly relativistic backflow around the pulsar into the trailing parts of the nebula. The time to cross a distance of $\sim 2d_s$ is $t_{\text{flow}} \approx 5 d_s c^{-1} \approx 10^7 \text{ s}$. Synchrotron cooling is no longer efficient, requiring $t_{\text{cool}} = 4.6 \times 10^9 \sigma_{\text{neb}}^{-3/4} \text{ s}$. Thus only a fraction

$t_{\text{flow}}/t_{\text{cool}} = 2 \times 10^{-3} \sigma_{\text{neb}}^{3/4}$ of the electron energy can be emitted as synchrotron radiation the region marked by the H α nebula. If a fraction f_8 of the pulsar spin-down luminosity is carried by the $\gamma \approx 10^8$ electrons, of which half are strongly shocked, the X-ray flux is

$$F_{\text{Neb}} \equiv f_{\text{Neb}} F_X \approx 1.5 \times 10^{-12} f_8^{7/4} D_{\text{kpc}}^{-2} \sigma_{\text{neb}}^{3/4} \text{ erg cm}^{-2} \text{ s}^{-1} \quad (2)$$

Our observations suggest $f_8 \leq 5 \times 10^{-2} I_{45}^{-7/4} D_{\text{kpc}}^{-2} \sigma_{\text{neb}}^{-3/4}$. As $t_{\text{flow}} \ll t_{\text{cool}}$, we predict faint diffuse X-ray emission with constant surface brightness to be present along a cylindrical trail formed as the relativistic pulsar wind expands into pressure equilibrium with the ISM behind the nebula. Instabilities occurring along the contact discontinuity separating shocked pulsar wind and shocked ISM lead to entrainment and mixing, with a consequent increase in the flow times in a boundary layer. Such processes make the constraint on f_8 even tighter.

The limits on f_5 and f_8 place added constraints on σ_p if these particles are to heat the photosphere of the companion star. Electrons with $\gamma \approx 10^8$ can explain the heating provided the pulsar wind is particle-dominated ($\sigma_p \leq 0.1$) all the way back to the companion. Alternatively, $\gamma \approx 10^5$ electrons might just suffice if they carry all of the particle energy flux in a magnetically dominated ($\sigma_p \approx 4$) wind. Otherwise, electrons with energies intermediate to these, or relativistic ions, carrying a fraction $0.3 L_{\text{psr}}$ are required. This conclusion is robust to distance uncertainties, as the ratios of these three fractions are independent of the assumed pulsar distance.

This first detection of X-rays from a millisecond pulsar of low magnetic field strength has constrained some of the possible components of the pulsar wind. Deeper Rosat observations of this system should confirm the presence or lack of orbital modulation, allow mapping of the pulsar wind nebula and possible detection of the X-ray trail behind the nebula, and perhaps even result in detection of pulsed emission. $\square$

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- Fruchter, A. S., Stinebring, D. R. & Taylor, J. H. *Nature* **333**, 237–239 (1988).
- Kulkarni, S. R. & Hester, J. J. *Nature* **335**, 801–803 (1988).
- Fruchter, A. S., Bookbinder, J., Garcia, M. R. & Bailly, C. D. *Nature* **359**, 303–304 (1992).
- Ruderman, M. & Sutherland, P. G. *Astrophys. J.* **196**, 51–72 (1975).
- Arons, J., in *Electron-Positron Pairs in Astrophysics* (eds Burns, M. L. et al.) 163–193 (AIP, New York, 1983).
- Michel, F. C. *Rev. mod. Phys.* **54**, 1–66 (1982).
- Kennel, C. F. & Coroniti, F. V. *Astrophys. J.* **283**, 710–730 (1984).
- Coroniti, F. V. *Astrophys. J.* **349**, 538–545 (1990).
- Ryba, M. F. & Taylor, J. H. *Astrophys. J.* **380**, 557–563 (1991).
- Pfeffermann, E. et al. *Proc. Soc. Photoopt. Instrum. Eng.* **733**, 519–532 (1986).

- Lasker, B. M. et al. *Astr. J.* **99**, 2019–2058 (1990).
- Kulkarni, S. R., Djorgovski, S. G. & Fruchter, A. S. *Nature* **334**, 504–506 (1988).
- Djorgovski, S. G. & Evans, C. R. *Astrophys. J.* **335**, L61–L65 (1988).
- Press, W. H., Flannery, B. P., Teukolsky, S. A. & Vetterling, W. T. *Numerical Recipes in C* 490–494 (Cambridge Univ. Press, 1988).
- Harnden, F. R. & Seward, F. D. *Astrophys. J.* **23**, 279–285 (1984).
- Phinney, E. S., Evans, C. R., Blandford, R. D. & Kulkarni, S. R. *Nature* **333**, 832–834 (1988).
- Arons, J. *Nature* **302**, 301–305 (1983).
- Hoshino, M., Arons, J., Gallant, Y. A. & Langdon, A. B. *Astrophys. J.* **390**, 454–479 (1992).

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X-rays from the eclipsing pulsar 1957+20

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TWO examples of eclipsing pulsars, PSRs 1957+20 and 1744-25, have been discovered and studied¹⁻⁷. In these systems, radiation from a pulsar drives a particle wind from a low-mass degenerate companion, creating an outflow which eclipses the radio pulsar once each orbital period. Ultimately the secondaries in both of these systems may be eroded away entirely, but the process by which the radiative wind ablates the smaller star remains controversial. Here we report the detection of soft X-rays, of ~1 keV energy, from PSR1957+20. This high-energy radiation, also observed by Kulkarni *et al.*⁸, should be a valuable diagnostic of the wind in this recycled pulsar system. Possible sources of the X-ray emission are the interstellar nebula driven by the pulsar wind, the interaction between the pulsar and its evaporating companion, and the pulsar itself. The small apparent size of the X-ray object argues against the first of these possibilities, and suggests that the X-rays are produced within the binary.

The primary star of the PSR1957+20 system is a pulsar of period 1.6 ms, which is eclipsed in the metre waveband for ~50 minutes out of each 9.16-hour binary orbit. Timing the pulsar allows the mass of the companion to be measured at ~0.025 solar masses ($M_{\odot}$), well below the hydrogen burning limit for ordinary main-sequence stars. The Roche lobe of this secondary is too small by a factor of two to account for the observed eclipse; this has led to the suggestion that the eclipse is created by a wind from the companion induced by the pulsar radiation field^{1,9-11}. This interpretation is supported by the observation of pulse timing delays near eclipse which suggested a free-electron column density in excess of 10^{17} cm^{-2} (refs 1-3). Further evidence of the strong effect of the pulsar radiation on the secondary is provided by optical observations¹²⁻¹⁵, which show luminosity variations of at least three magnitudes with a maximum when the pulsar-illuminated side of the companion faces the Earth, and by a measurement of the orbital period derivative³, which suggests that the companion may be completely evaporated in less than 10^8 years.

One of the main uncertainties in the increasingly complex models being developed for PSR1957+20 and the similar source PSR1744-25 is the nature of the high-energy radiation fields

TABLE 1 HRI observations of PSR1957+20

Date (UT)	Time	Integration length(s)	Counts
4 May 1991	11:52-13:26	2,781	4
4 May 1991	23:44-01:51	3,438	1
5 May 1991	04:43-08:22	3,732	1
5 May 1991	11:05-13:25	4,877	5

The dates and times of the four HRI observations, the effective integration times and the number of photons detected in a circular aperture with radius 6" about the pulsar position. Where the observations extend across midnight, the date reflects the start time. Rosat observations are occasionally interrupted by Earth occultation or passage through the South Atlantic Anomaly. Thus the effective integration time can be much shorter than the spacecraft observing time.

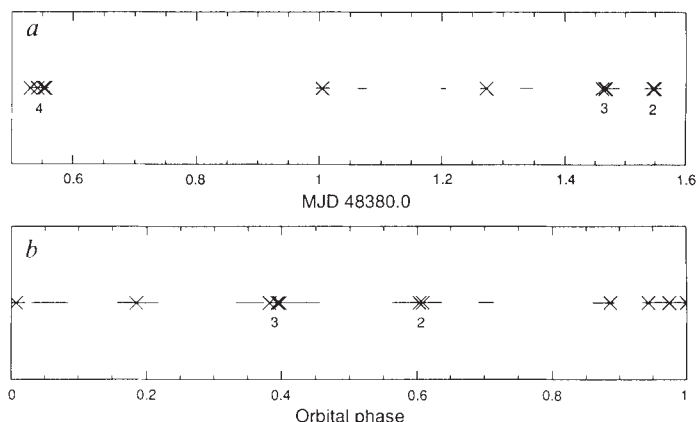
from such 'recycled' pulsars, and the exact mechanism(s) by which the radiation induces a wind from the companion. Soft (~1-keV) X-rays, megaelectronvolt γ -rays and high-energy particles have been invoked as the relevant radiation, with correspondingly different wind production mechanisms¹⁶⁻²⁰. Here we report the discovery in Rosat data of a soft X-ray flux from PSR1957+20. This may prove important for understanding the physics of pulsar emission and the origin of the wind from the companion.

The possibility that X-rays from PSR1957+20 could be responsible for the evaporation of its companion, or that hot ablated gas could emit in the X-ray regime, led us to observe this object with the Rosat (Roentgen satellite) X-ray telescope. Rosat has two focal-plane instruments capable of this observation, the Position Sensitive Proportional Counter (PSPC) and the High Resolution Imager (HRI)²¹. Although the PSPC is, for a broad range of spectral signatures, two to three times as sensitive as the HRI, its spatial resolution is only ~20", whereas the resolution of the HRI is closer to 5". Our concern that flux from an extended interstellar nebula about the pulsar²² could be confused with that from the binary led us to observe PSR1957+20 with the HRI.

Table 1 lists the start and stop times of each of our four observing sessions, the effective integration times and the number of photons detected within a circle of radius 6" of the pulsar position. The times and orbital phases of arrival of these photons are shown in Fig. 1.

The average background in a circle of 6" radius was ~1.5 counts during this observation. The 11 counts recorded at the position of the pulsar therefore represent a $\geq 7\sigma$ detection of X-ray emission. Only three other sources of similar or greater flux were detected in the 0.4-square-degree HRI image. The probability that the source at the position of the pulsar is an unrelated background object is therefore $\leq 10^{-5}$.

FIG. 1 The arrival times of the photons detected in these HRI observations. *a*, Photon arrival times (X) plotted against modified Julian date (MJD). *b*, Photon arrival times plotted against the orbital phase of PSR1957+20. Phase $\phi=0$ is the ascending node of the pulsar orbit; phase $\phi=0.25$ is conjunction and the centre of eclipse. (See ref. 3 for the most recently published ephemeris.) The horizontal bars mark times when the HRI was integrating. Where the arrival times are bunched, the total number of photons in a group is noted.



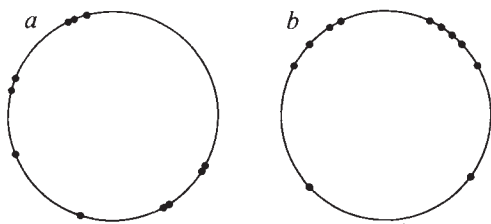


FIG. 2 Arrival times of the photons folded at the pulsar period (a) and half the pulsar period (b). The arrival times have been reduced to the Solar System barycentre and the orbital motion of the pulsar has been subtracted before folding. The circumference of the circle represents the ~ 1.607 -ms period of PSR1957+20 in a, and half this period in b. Although the relative arrival times of the photons may be as accurate as 0.1 ms, the absolute timing accuracy of the Rosat clock is only about 1 ms; therefore, the orientation of the circles is not significant.

The HRI in conjunction with the Rosat mirror assembly has an effective cross-section of $\sim 80 \text{ cm}^2$ for photons between 0.8 and 1.6 keV, with little or no effective area below 0.5 keV or above 2 keV. Using the best available measurements of the HRI focal-plane point spread function²³, we estimate that $\sim 75\%$ of the photons from the source incident on the HRI lie within our $12''$ aperture. Therefore the count rate seen here corresponds to an incident flux at the Earth of $\sim 2 \times 10^{-14} \text{ erg s}^{-1} \text{ cm}^2$ in the energy range of 0.8 to 1.6 keV, and a source luminosity in this energy band, for either a flat or Crab-like spectrum, of $\sim 2 \times 10^{30} (1 - \alpha)^{-1} (D/1 \text{ kpc})^2 \text{ erg s}^{-1}$, where α is the fraction of photons absorbed by the interstellar medium, and D is the distance to the pulsar. The total H I column density²⁴ of $2.5 \times 10^{21} \text{ cm}^{-2}$ in the direction of PSR1957+20 implies that $\alpha \approx 0.5$ (ref. 24). For an assumed distance to the pulsar of 1.5 kpc (refs 4, 26), the total luminosity is then $\sim 10^{31} \text{ erg s}^{-1}$ in the observed bandpass. Given the small number of photons detected, the 1σ errors in our flux and luminosity estimates are $\sim 30\%$.

The distribution of counts in the separate observing sessions is suggestive of source variability, an impression which remains if the photons are grouped according to orbital phase (see Fig. 1). Indeed, 10 of the 11 photons are found nearer to quadrature than to eclipse or anti-eclipse. When these statistics are combined with the phase distribution of the observing time, we find that the magnitude of variability seen here will occur by chance with a probability of $\sim 20\%$.

The H α nebula surrounding PSR1957+20 covers about 0.5 arcmin^2 of the sky. We have therefore also extracted the photons from this region to determine whether the nebula is an extended X-ray source. After eliminating the 11 photons found in the detect cell of the pulsar, the region covered by the nebula yields fewer counts than the expected background. Using the distance and absorption described above, we find a 3σ upper limit to any extended emission in the 0.8 to 1.6 keV band of $\leq 10^{31} \text{ erg s}^{-1}$. The H α emission is nearly $1'$ across whereas our resolution is about $5''$; the X-ray source is unresolved, however, and its position agrees with the radio timing position of the pulsar to $1''$, well within the $3''$ Rosat pointing errors (Rosat team, personal communication). Therefore the X-ray emission is concentrated in only a few per cent of the projected area of the H α nebula, so we think it unlikely that the nebula is the X-ray source.

The X-ray luminosity reported here represents only $\sim 10^{-4}$ of the pulsar spin-down power and therefore could be produced directly by the neutron star. If the pulsar were the immediate source, we would expect the emission to be constant on time-scales comparable to the orbital period, since the general transparency of the companion wind to 20-cm radio waves implies a free-electron column density $\leq 10^{20}$, which is far too small to significantly attenuate keV photons unless the evaporate is, contrary to all expectation^{2,4}, largely neutral, or possesses a metallicity nearly 50 times solar. But the radiation might well be modulated on the radio pulsation period. We have therefore reduced the photon arrival times to the Solar System barycentre using the standard Rosat processing software, PROS, and have then adjusted these arrival times to remove the orbital motion of the pulsar. The relative accuracy of the times thus calculated is no better than 0.1 ms, and could be worse if the drift of the corrected Rosat clock is larger than expected. Figure 2a shows the arrival times plotted on a circle representing the pulse period; there is little evidence for pulsations at this period. Radio pulsars can, however, display a double peaked profile in the X-ray; therefore, we have also plotted the photons folded at twice the pulsar spin rate (Fig. 2b). Although this latter plot may show a hint of periodicity, any decisive test of pulsation will require many more photons than are reported here.

Another explanation of the observed X-rays may be more likely. The energy we see emitted in soft X-rays corresponds to $\sim 10\%$ of the energy reprocessed by the companion in the optical^{2,4} or $\sim 4\%$ of the pulsar spin-down energy incident on the companion's Roche lobe. It seems likely that the shocked pulsar wind surrounding the companion will radiate in the X-ray^{10,18,27}; indeed, Arons and Tavani²⁷ predict a soft X-ray luminosity similar to that observed here. This radiation could in turn be responsible for the ablation of the companion^{10,18}. The X-ray emission would then vary with orbital phase, although instabilities in the interaction of the companion and pulsar winds could also produce flaring. The sense of the modulation should help to indicate the geometry of the interaction between the pulsar radiation and the companion wind. Substantially deeper X-ray observations may be able to determine whether this source is variable at the spin or orbital period, and thus establish whether the emission comes directly from the neutron star or from the ablation of the secondary. $\square$

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- Fruchter, A. S., Stinebring, D. R. & Taylor, J. H. *Nature* **333**, 237–239 (1988).
- Fruchter, A. S. *et al. Astrophys. J.* **351**, 642–650 (1990).
- Ryba, M. F. & Taylor, J. H. *Astrophys. J.* **380**, 557–563 (1991).
- Fruchter, A. S. & Goss, W. M. *Astrophys. J.* **384**, L47–L51 (1992).
- Lyne, A. G. *et al. Nature* **347**, 650–652 (1990).
- Nice, D. J., Thorsett, S. E., Taylor, J. H. & Fruchter, A. S. *Astrophys. J.* **361**, L61–L63 (1990).
- Thorsett, S. E. & Nice, D. J. *Nature* **353**, 731–733 (1991).
- Kulkarni, S. R., Phinney, E. S., Evans, C. R. & Hasinger, G. R. *Nature* **359**, 300–302 (1992).
- Kluźniak, W., Ruderman, M., Shaham, J. & Tavani, M. *Nature* **334**, 225–227 (1988).
- Phinney, E. S., Evans, C. R., Blandford, R. D. & Kulkarni, S. R. *Nature* **333**, 832–834 (1988).
- van den Heuvel, E. P. J. & van Paradijs, J. *Nature* **334**, 227–228 (1988).
- van Paradijs, J. *et al. Nature* **334**, 684–686 (1988).
- Fruchter, A. S., Gunn, J. E., Lauer, T. R. & Dressler, A. *Nature* **334**, 686–689 (1988).
- Djorgovski, S. & Evans, C. R. *Astrophys. J.* **335**, L61–L65 (1988).

- Callanan, P. J., Charles, P. A. & van Paradijs, J. *Mon. Not. R. astr. Soc.* **240**, 31p–34p (1989).
- Ruderman, M., Shaham, J. & Tavani, M. *Astrophys. J.* **336**, 507–518 (1989).
- Ruderman, M., Shaham, J., Tavani, M. & Eichler, D. *Astrophys. J.* **343**, 292–312 (1989).
- Cheng, A. F. *Astrophys. J.* **339**, 291–296 (1989).
- Krolik, J. H. & Sincell, M. W. *Astrophys. J.* **357**, 208–215 (1990).
- Eichler, D. *Mon. Not. R. astr. Soc.* **254**, 11p–13p (1992).
- Pfeffermann, E., *et al. SPIE* **733**, 519–532 (1986).
- Kulkarni, S. R. & Hester, J. J. *Nature* **335**, 801–803 (1988).
- Rosat Calibration Team *ROSAT Status Rep. No. 22* (1992).
- Weaver, H. & Williams, D. R. W. *Astr. Astrophys. Suppl. Ser.* **17**, 1–249 (1974).
- Morrison, R. & McCammon, D. *Astrophys. J.* **270**, 119–122 (1983).
- Aldcroft, T. L., Romani, R. W. & Cordes, J. M. *Astrophys. J.* (in the press).
- Arons, J. & Tavani, M. *Astrophys. J.* (in the press).

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Star formation and the origin of stellar masses

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SIGNIFICANT progress has been made in recent years in the imaging of star-forming regions and in the theoretical modelling of the process of star formation¹, but the physical process that determines the mass spectrum of stars remains unclear. Here we propose a model in which the protostar phase ends when the protostar embedded in a condensing core of molecular gas interacts with another protostar or star, and is ejected from its core. Such interactions must be important if stars preferentially form in dense but ultimately unbound protoclusters. In a simple model in which protostars accrete at a constant rate, the final distribution of stellar masses asymptotically approaches a simple universal distribution which is very similar to the observed mass function of stars. The general form of the mass function in this model is determined by a competition between accretion and collision rates, which provides a qualitative explanation for the differences in star formation in different environments (such as the galactic disk, globular clusters and the galactic halo).

Two important aspects of the star-formation process which are not well understood are what determines the transition from the embedded protostar phase to the T Tauri phase and the related question of what process determines stellar masses and hence the initial mass function (IMF). Strong, bipolar T Tauri winds have been suggested as a mechanism to terminate the protostar accretion phase (see, for example ref. 1), but such a mechanism has not been demonstrated convincingly. In addition, detailed studies of young stellar objects² (YSOs) reveal that objects in different pre-main-sequence phases are well mixed spatially within their star-forming environment, as well as in the Hertzsprung–Russell diagram (in particular near the stellar birthline³). Because the location in the Hertzsprung–Russell diagram uniquely determines the evolutionary phase of a star, this suggests that the transition is caused by an external agent, and not by an intrinsic, evolutionary effect such as ignition of a particular nuclear fuel or exhaustion of the circumstellar envelope. (An internal random process such as random bipolar wind properties, random initial conditions or duplicity might also explain these observations.) Most attempts to derive the IMF have concentrated on determining the mass spectrum of cores resulting from the fragmentation and coagulation of gravitationally unstable clouds or clumps (for a review see ref. 4). In these studies, the final mass spectrum of stars is essentially determined by the mass spectrum of the cores from which they formed (although not necessarily linearly, as not all the matter in the cores need become part of the star and the initial mass spectrum can be modified by subsequent accretion^{5,6}). In our model, these and several other important processes are not explicitly included. Although even our simple model can explain most of the features of the observed IMF, we emphasize that, in a comprehensive theory of star formation, these other processes will have to be taken into consideration.

Recent observations of star-forming regions^{7–9} suggest that low-mass stars preferentially form in dense clusters. In this case, mutual interactions between YSOs are necessarily important. We therefore propose that protostars accrete from their parent cores until they are ejected from their cores because of a collision with another object in the star-forming cloud or clump. The final mass of a protostar is then mainly determined by the time it took for a collision to take place and not by the mass in the core from which the star formed (the final star mass can be larger than the initial mass in the core, if the core continues to accrete from its environment). Many of our later conclusions may apply to other dynamical processes (frictional drag¹⁰, exter-

nal impulses¹¹) which may also be effective in separating protostars from their envelopes and in exposing them as young stars (T Tauri stars). Here we consider two more direct mechanisms, namely collisions between two protostellar cores (protostars embedded in dense envelopes from which they accrete) and collisions between a protostellar core and a star (a YSO that is no longer embedded in a massive envelope; this definition includes T Tauri stars). The cross-section for collisions between protostellar cores is mainly determined by the geometrical size of their envelopes. The outcome of such collisions depends on the relative velocity of the colliding cores. The envelopes can either be disrupted, exposing the bare protostars, or merge to form a single core. Even in the latter case, the protostars will receive a kick velocity relative to the merged envelopes which is of the order of the initial relative velocity. We find that, if the initial relative velocity is sufficiently supersonic (as is likely¹²), this kick velocity will exceed the central escape velocity and the protostars will therefore escape from any core remnant.

A collision between a protostellar core and a star will also separate a protostar from its envelope, if the kick velocity imparted to the protostar (relative to its envelope) by a star passing through the core exceeds the central escape velocity. We have estimated the differential gravitational impulse imparted to the central stellar seed relative to its envelope for characteristic envelope density profiles¹³ ($\rho \propto r^{-\gamma}$; $\gamma \approx 1.5$) and core sizes¹³ ($R_0 \approx 0.05$ pc). We find that the cross-section for collisions that eject the stellar seeds is $\sim 10\%$ of the geometrical cross-section of the core. In the simulations below, direct core-core collisions dominate initially, until the number of stars significantly exceeds the number of protostellar cores.

To illustrate how a collisional model leads to a mass spectrum, we adopt a simple model for the star-formation process. We assume that protostellar cores form at a constant rate (per unit gas mass), α , and that protostars accrete from their cores at a constant mass accretion rate, $\dot{M}$, until they are ejected (the individual rates for core-core collisions and core-star collisions are β and βz [where $z \approx 0.1$], respectively). The value of α is uncertain and depends on the mechanism that regulates the star-formation process. From global considerations of star-formation efficiencies in the Galaxy, we estimate that $\alpha \approx 10^{-7} - 10^{-9} M_{\odot}^{-1} \text{ yr}^{-1}$, where $M_{\odot}$ is the solar mass (also see ref. 14). $\dot{M}$ is mainly determined by the sound speed, c_s , in the gravitationally unstable envelope^{15,16} ($\dot{M} \approx c_s^3/G$, where G is the gravitational constant). For cool clouds (with temperatures $T \approx 10$ K), $\dot{M} \approx 10^{-5} - 10^{-6} M_{\odot} \text{ yr}^{-1}$. The core-core collision rate per pair is given by $\beta \approx \langle \sigma v \rangle / V$, where $\sigma \approx 0.008 \text{ pc}^2$ is the geometrical cross-section of a protostellar core, $v \approx 1 \text{ km s}^{-1}$ is the velocity dispersion of cores, and V is the volume within which collisions occur.

We have solved the differential equations that describe this simple model for the star-formation process by standard numerical methods. In Fig. 1, we present some of the resulting IMFs, which may be representative for different star-forming environments (a clump in a molecular cloud, a bound and a failed globular cluster, respectively). Details will be presented elsewhere; N.M.P. and P.P., manuscript in preparation). One of the main results of our calculations (which follows from straightforward asymptotic analysis) is that the IMF asymptotically approaches a simple and universal distribution, provided that star formation is relatively inefficient (which here means that the timescale on which the gas in a star-forming cloud is locked up in stars is longer than the collision time between protostellar cores and stars). This asymptotic solution is of the form

$$f(m) dm \propto \frac{1 - (1+m)e^{-m}}{m^2} g dm \quad (1)$$

where $m \equiv M/M_{\text{sc}}$ is a dimensionless mass and $g \equiv \exp[-(M/M_{\text{co}})^2]$ is an exponential cut-off factor (M is the mass of the star, and M_{sc} and M_{co} are the scaling mass and the cut-off mass, respectively). Qualitatively, this distribution

behaves like an inverse-square power law (for $M_{sc} < M < M_{co}$) which at low masses has a smooth turnover and at high masses has a sharp cut-off (see the curve labelled 'globular cluster' in Fig. 1). In the range in which the distribution follows a power law, it closely resembles a Salpeter IMF¹⁷ ($f_{\text{Salpeter}} \propto m^{-2.35}$, shown for comparison in Fig. 1). The mass at which the distribution turns over scales with M_{sc} ($M_{sc} \approx \dot{M}/[\beta\alpha M_g t]$, where M_g is the total mass in the star-forming cloud or clump and t is the star-formation time). Physically, M_{sc} can be written as the product of the mass accretion rate multiplied by a characteristic, instantaneous collision time. Because the collision time decreases with time as the number of scatterers increases, this characteristic mass decreases with time. Thus, the distribution more closely approaches a simple power law to lower masses, the longer star formation proceeds. The cut-off at large masses is determined by competition between the rate at which new protostars are formed and the rate at which they are destroyed by collisions. The cut-off mass, M_{co} , can be written approximately as $\dot{M}/\sqrt{\beta\alpha M_g}$, where the denominator corresponds to the geometric mean of the collision rate per pair and the protostar formation rate. The examples in Fig. 1 illustrate how the mass

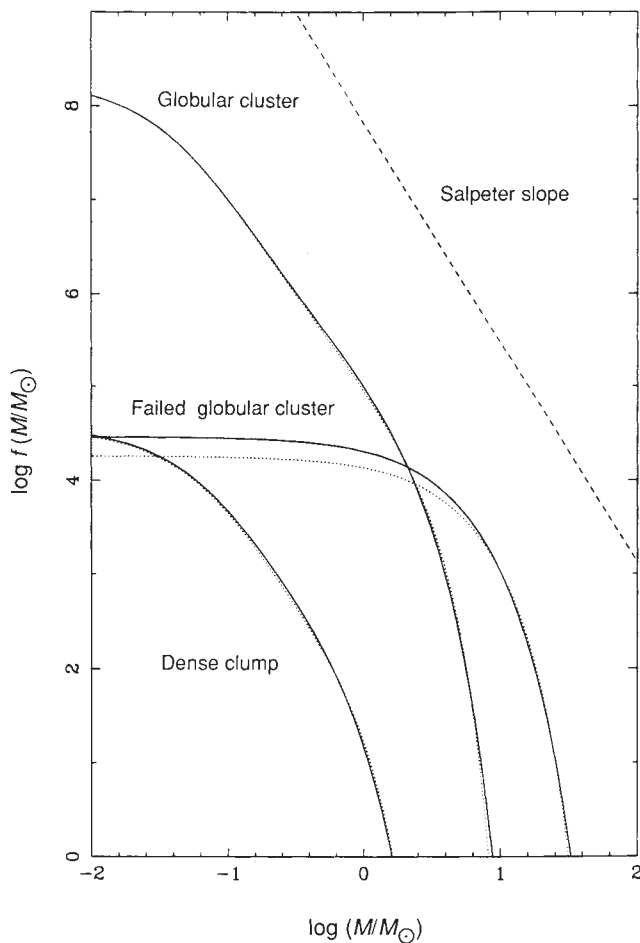


FIG. 1 Initial mass functions resulting from a collisional model and illustrating three different star-formation environments: a globular cluster, a 'failed' globular cluster and a dense clump in a molecular cloud, respectively (solid curves as indicated). The 'globular cluster' and the 'clump' IMFs both have extended power-law segments, resembling a Salpeter IMF (shown, for comparison, as a dashed curve). The initial gas masses, M_g , in the three cases were 10^6 , 10^6 and $5 \times 10^2 M_\odot$, the star-formation rates, α , were 10^{-7} , 10^{-8} and $10^{-7} M_\odot^{-1} \text{yr}^{-1}$; the collision rates, β , were 10^{-9} , 5×10^{-10} and $2 \times 10^{-7} \text{yr}^{-1}$ ($z=0.1$ in all cases); the protostar accretion rates, $\dot{M}$, were 10^{-5} , 10^{-5} and $10^{-6} M_\odot \text{yr}^{-1}$. At the end of the simulations (after 10^6 , 10^7 and $5 \times 10^7 \text{yr}$, respectively), the gas mass fractions were ~ 50 , ~ 70 and $\sim 70\%$, respectively. For comparison, the dotted curves represent the asymptotic solutions given in equation (1).

distributions vary for different input parameters reflecting different stellar environments. In a more realistic model, α , β and $\dot{M}$ probably have to vary both spatially and temporally. In addition, the variation of core sizes and the dynamical evolution of the star-forming region have to be taken into account. Although this will undoubtedly alter this simple distribution law, the very existence of a universal, asymptotic solution may be sufficient to guarantee that the overall distribution (averaged over various populations) will still reflect the characteristics of this solution, which is qualitatively very similar to the Salpeter IMF¹⁸.

In addition to making quantitative predictions about initial mass distributions, such a star-formation scheme (or variation thereof) may also explain the different apparent formation efficiencies and biases in the IMF in different environments. For example, it has been argued¹⁹ that star-formation in globular clusters must be very efficient and occur essentially on a dynamical timescale (unlike star formation in the galactic disk) to avoid self-pollution with heavy elements produced in the first supernovae (leading to large abundance spreads, which are not observed²⁰). In our scheme, this is no longer necessary, as globular clusters constitute a very high (internal) collision environment, in which massive stars are unable to form ('globular cluster' in Fig. 1). The only constraint is that the cut-off mass, M_{co} , is larger than $\sim 4 M_\odot$, to produce the population of neutron stars observed in globular clusters, but less than $\sim 12 M_\odot$, to avoid self-pollution (core-collapse supernovae with progenitor masses $\leq 12 M_\odot$ do not eject significant amounts of heavy elements²¹). Furthermore, we expect that star-forming clouds that are able to form massive stars ('failed globular cluster' in Fig. 1) will become unbound because of the first generation of massive stars and their supernovae. If failed clusters are the environment in which halo stars and population III stars are formed, their IMFs should be relatively flat; that is, biased towards high masses (Fig. 1). Massive globular clusters like ω Centauri and M22 which show internal abundance spreads²⁰ would be intermediate cases.

Our star-formation scheme may also apply to massive stars (although other effects such as supernovae, energetic stellar winds and photoionization will introduce further complications²²). The main difference may be that they form in dense, but relatively warm clouds, which leads to higher accretion rates and longer collision times (both effects tend to produce a larger average mass). In addition, star formation in the outskirts of cold clumps or between cold clumps also favours the formation of massive stars. These massive stars, or individual massive stars that sink to the centre of cold clumps (possibly while they are still forming), are likely to affect them considerably and eventually to stop low-mass star formation in these cold clumps (for example, by heating the cloud or by disrupting it²³). This suggests a sequential picture for the star-formation process. Low-mass stars form first in cold clumps in a molecular cloud, until these clumps are destroyed by the first generation of massive stars which form in their neighbourhood. The later phases of star formation (which are generally the more spectacular and better studied phases) will be dominated by the formation of massive stars, surrounded by (unbound) clusters of previously formed low-mass stars (see also refs 10, 24).

Our model may also be relevant to the formation of binary and multiple systems, because core-star collisions in which the protostar is dislodged from the centre of its core but remains bound to it should be much more common than collisions that eject the protostar. After a protostar has been displaced from the centre of a core, a new protostar may form in the centre. The displaced, but bound, protostar will spiral back to the centre (on a frictional timescale of $\sim 10^4$ – 10^5yr) and ultimately either form a binary (or multiple system if there are multiple collisions) or coalesce with the new protostar (see also refs 11, 25).

Although many of our conclusions should be considered preliminary (mainly because several other processes need to be

taken into account), we conclude that collisional effects are important in the star-formation process, provided only that stars form preferentially in dense clusters. Independent of the details

of the model, such dynamical interactions can profoundly affect issues such as IMFs, star-formation efficiencies and multiple systems. □

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1. Shu, F. H., Adams, F. C. & Lizano, S. A. *Rev. Astr. Astrophys.* **25**, 23–81 (1987).
2. Bertout, C. A. *Rev. Astr. Astrophys.* **27**, 351–395 (1989).
3. Stahler, S. *Astrophys. J.* **332**, 804–825 (1988).
4. Cayrel, R. in *Physical Processes in Fragmentation and Star Formation* (eds Capuzzo-Dolcetta, R., Chiosi, C. & Di Fazio, A.) 343–355 (Kluwer, Dordrecht, 1990).
5. Silk, J. & Takahashi, T. *Astrophys. J.* **229**, 242–256 (1979).
6. Smith, G. H. *Astrophys. J.* **293**, 251–257 (1985).
7. Herbig, G. H. & Terndrup, D. M. *Astrophys. J.* **307**, 609–618 (1986).
8. Strom, K. M., Margulis, M. & Strom, S. E. *Astrophys. J.* **346**, L33–L35 (1989).
9. Lada, E. A., DePoy, D. L., Evans, N. J. II & Gatley, I. *Astrophys. J.* **371**, 171–182 (1991).
10. Myers, P. C. in *The Formation and Evolution of Star Clusters* (ed. Janes, K.) 73–77 (Astr. Soc. Pacific, San Francisco, 1991).
11. Pringle, J. E. *Mon. Not. R. astr. Soc.* **239**, 361–370 (1989).
12. Solomon, P. M., Rivolo, A., Barrett, J. & Yahil, A. *Astrophys. J.* **319**, 730–741 (1987).
13. Fuller, G. A. & Myers, P. C. *Astrophys. J.* **384**, 523–527 (1992).
14. McKee, C. F. *Astrophys. J.* **345**, 782–901 (1989).

15. Larson, R. B. *Mon. Not. R. astr. Soc.* **145**, 271–295 (1969).
16. Shu, F. H. *Astrophys. J.* **214**, 488–497 (1977).
17. Salpeter, E. E. *Astrophys. J.* **121**, 161–167 (1955).
18. Scalo, J. M. *Fund. Cosmic Phys.* **11**, 1–278 (1986).
19. Murray, S. D. & Lin, D. N. C. *Astrophys. J.* **339**, 933–942 (1990).
20. Kraft, R. P. A. *Rev. Astr. Astrophys.* **17**, 309–343 (1979).
21. Nomoto, K. in *The Crab Nebula and Related Supernova Remnants* (eds Kafatos, M. C. & Henry, R. B. C.) 97–113 (Cambridge Univ. Press, 1985).
22. Genzel, R. in *The Physics of Star Formation and Early Stellar Evolution* (eds Lada, C. J. & Kylafis, N. D.) 155–219 (Kluwer, Dordrecht, 1991).
23. Bally, J. & Lada, E. in *The Formation and Evolution of Star Clusters* (ed. Janes, K.) 35–39 (Astr. Soc. Pacific, San Francisco, 1991).
24. Garmany, C. D. in *The Formation and Evolution of Star Clusters* (ed. Janes, K.) 23–34 (Astr. Soc. Pacific, San Francisco, 1991).
25. Clarke, C. J. & Pringle, J. E. *Mon. Not. R. astr. Soc.* **249**, 588–595 (1991).

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Resolution of magnetic flux tubes on the Sun

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MAGNETIC flux at the surface of the Sun is predominantly concentrated in discrete areas with kilogauss field strengths¹. Except for sunspots, these areas are too small to have been resolved by conventional observations. These magnetic flux tubes are an essential part of the physics of the activity and heating of the outer atmosphere of the Sun and other late-type stars², but although their average properties have been studied in considerable detail^{3,4}, direct observations of them have been lacking because of turbulence in the Earth's atmosphere, which limits resolution to ~400 km. Using a newly developed technique of speckle interferometry⁵, we have obtained simultaneous direct observations of the white-light and magnetic field signature of flux tubes. Individual flux tubes are seen, with resolved diameters of ~200 km and continuum brightness contrast of at least +30%. Magnetic features larger than 300 km in size tend, however, to be darker than their surroundings.

More than 90% of the magnetic flux outside sunspots is concentrated into small flux tubes with field strengths of 1–2 kG (ref. 6). Because these flux tubes have not yet been spatially resolved, our knowledge of their evolution, dynamics and morphology is limited. White-light bright points seen in active regions and in the network have often been associated with these magnetic flux tubes^{7–9}. But direct observations showing these bright points to be related to concentrated magnetic fields are lacking because of the limited spatial resolution of conventional magnetograms (maps of the Zeeman-induced circular polarization, which is roughly proportional to the line-of-sight magnetic flux). We have overcome the resolution limit imposed by optical disturbances of the Earth's atmosphere by using a method based on speckle interferometry⁵. It provides diffraction-limited, simultaneous magnetograms and white-light images.

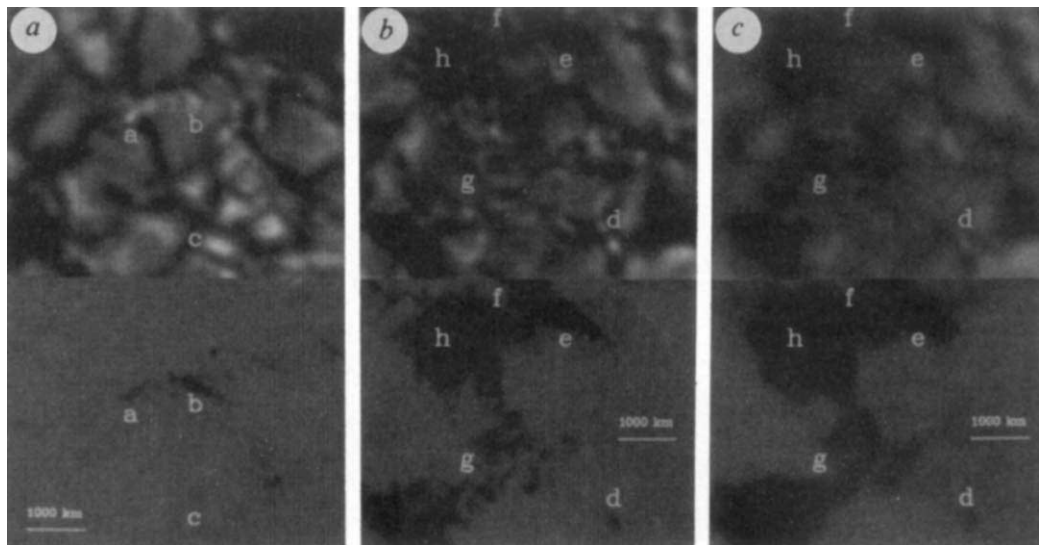
The observations were recorded with the 50-cm Swedish Vacuum Solar Telescope at La Palma (Canary Islands) near the centre of the solar disc. The white-light images were recorded through a filter with a full width at half maximum (FWHM) of 82 Å around 5,200 Å, whereas the magnetograms were recorded at wavelength 5,250.2 Å (Fe I) with 0.15 Å FWHM. There the effective point spread function of the speckle reconstruction has a FWHM of 140 km.

Figure 1a shows magnetic elements near a weak plage. The granules around the bright point 'a' show the typical daisy-like arrangement found in the presence of network bright points¹⁰. Point 'a' has a maximum continuum contrast (relative deviation from the average intensity) of 34%. Together with its extension towards the right, it is cospatial with a magnetogram signal of up to 4%. The magnetic element 'b', whose strongest magnetogram signal of 6% coincides with a bright region (continuum contrast 31%), is situated at the border of a granule. Although there is some signal in other parts of the magnetogram, it is not significantly above the noise level.

A more active region is shown in Fig. 1b. Within the pores the signal-to-noise ratio is too small to give reliable magnetogram signals. The bright point 'd' (continuum contrast 30%) is cospatial with a strong magnetogram signal of up to 8%. The FWHM is 180 (±20) km in the continuum as well as in the magnetogram. An empirical flux tube model¹¹ with field strength 2,360 G at the level of continuum formation and a contrast of 30% produces a maximum magnetogram signal of 10%, once the spectral smearing due to the instrument has been taken into account. The magnetic field is therefore almost fully resolved there, although it is likely that the white-light structure is not resolved. The associated magnetic flux is of the order of 5×10^{17} maxwell (Mx). Element 'd' is not seen as a bright point or as concentrated magnetic flux in the data recorded 8 minutes before and 8 minutes afterwards. There is, however, a weak magnetogram signal slightly above the noise level at the same location. The raw images show the same behaviour, making it unlikely that the change is due only to the speckle reconstruction. The other magnetic features also show considerable changes. The average magnetic flux, however, is much more stable.

The dark magnetic feature just above 'e' with a continuum contrast of -31% has a magnetogram signal of up to 12%. When the temperature sensitivity of the spectral line is accounted for, this signal is also consistent with an almost fully resolved magnetic field. Although the magnetic field to the right of 'f' and below and to the right of 'g' seems to be located in individual elements, there is no particular white-light signature associated with the magnetogram signal. The average continuum contrast there is -6%. About 50% of the area there is covered with magnetic fields if we assume that the fields have field strengths of ~2kG at the level of continuum formation. The abnormal granulation¹² in this highly magnetic region cannot be adequately described by small granules embedded in a net of darker lanes¹³; on the contrary, we see no morphological difference between bright and dark features at the present spatial resolution.

FIG. 1 *a*, White-light image (upper panel) and the magnetogram in the blue wing of the Fe I line at 5,250.2 Å (lower panel) of solar magnetic elements near a weak plage. In the magnetogram, grey corresponds to zero signal, and black and white correspond to opposite polarities. The observations were recorded with the 50-cm Swedish Vacuum Solar Telescope at La Palma (Canary Islands) under favourable seeing conditions near the centre of the solar disc. A speckle interferometric technique was used to remove the disturbances caused by turbulence in the Earth's atmosphere⁵. Two cameras recorded simultaneous images in a white-light channel and in a narrow-band channel. The speckle imaging reduction of the data in the white-light channel is used to calculate the optical transfer function of each individual image. This transfer function is then used to deconvolve each simultaneous image in the narrow-band channel where the circular polarization is measured. This process takes into account the combined modulation transfer function of the telescope and the atmosphere. *b*, As *a* in a pore region. The grey scale of the magnetogram is different from *a* to account for the larger magnetogram signals here. Within the pores, the signal to noise ratio is too small to give reliable results in the magnetograms. The bright point labelled 'd' is an



almost fully resolved flux tube with a diameter of ~ 180 km. *c*, Unrestored images from the same data set as *b*. The sharpest single white-light image is shown in the upper panel, and the lower panel shows the average magnetogram signal after correction for image motion of the individual magnetograms. The contrast of the magnetogram has been increased with respect to *b*. Element 'd' of *b* is seen in both images, although at lower resolution and contrast. No sizable artificial magnetogram signal is generated by the speckle reconstruction.

In the data presented here and in different observations of the same kind, other details have been identified. (1) Near magnetic areas, there exist small granules (400–600 km diameter) with large continuum contrast (up to 40%) that have no associated magnetogram signal. An example is the granule to the right of 'c' in Fig. 1*a*. (2) The magnetic field of pores extends beyond the dark umbra. There the white-light images often show radially elongated structures similar to penumbral filaments in sunspots (for example at the left border of pore 'h' in Fig. 1*b*). (3) There exist umbral dots within pores having average photospheric continuum intensity (for example just to the right of 'h' in Fig. 1*b*).

Our results strongly depend on the reliability of the speckle restoration. Its reliability was tested by restoring independent data sets extracted from the same image series. The white-light signals differ on average by $\sim 0.5\%$ and the magnetogram signals by 1%. The reality of the features in the restored images is reconfirmed by their presence in unrestored images shown in Fig. 1*c*.

We draw the following conclusions from our investigation. (1) There exist continuum bright points with a contrast of at least 30% that are cospatial with strong magnetic fields. This contrast is very similar to that of network bright points¹⁴. The observed characteristics of these magnetic elements are compatible with the values predicted for flux tubes by indirect observations⁴ and theoretical models¹⁵. Our observations are therefore consistent with the assumption that some facular and network bright points are the white-light signature of magnetic flux tubes. But there exist other magnetic elements without bright points, as well as bright features without magnetic signal. (2) The individual magnetic flux tubes do not cover a significantly larger area than the cospatial bright points. Some observations seemed to show that the magnetic field occupies a much larger area than the bright points¹⁶, but this is probably due to the lower spatial resolution of the magnetic field data as compared with the continuum data. Because the diameter of the elements is

near the diffraction limit of the telescope, we cannot ascertain their shape and exact extent. (3) Magnetic elements larger than ~ 300 km in at least one dimension are mostly darker than the average intensity. Their properties are similar to 'magnetic knots'¹⁷ or 'invisible sunspots'¹⁸. The transition from small bright elements to larger dark elements is consistent with earlier observations¹⁹ and theoretical models²⁰. Our data suggest that the transition between bright and dark elements occurs at diameters of ~ 300 km. (4) In highly magnetic areas there is no clear relationship between continuum intensity and magnetogram signal at the smallest spatial scales. This agrees with other high resolution observations¹³. (5) Magnetic flux tubes may evolve noticeably within timescales of 15 minutes, in agreement with the timescales associated with bright points^{8,12}. □

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1. Stenflo, J. O. *Solar Phys.* **32**, 41–63 (1973).
2. Spruit, H. C. & Roberts, B. *Nature* **304**, 401–406 (1983).
3. Stenflo, J. O. *Astr. Astrophys. Rev.* **1**, 3–48 (1989).
4. Solanki, S. K. in *Solar Photosphere: Structure, Convection, and Magnetic Fields* IAU Symp. 138 (ed. Stenflo, J. O.) 103–120 (Kluwer, Dordrecht, 1990).
5. Keller, C. U. & von der Lüh, O. *Astr. Astrophys.* **261**, 321–328 (1992).
6. Frazier, E. N. & Stenflo, J. O. *Solar Phys.* **27**, 330–346 (1972).
7. Mehlretter, J. P. *Solar Phys.* **38**, 43–57 (1974).
8. Müller, R. *Solar Phys.* **85**, 113–121 (1983).
9. Schüssler, M. & Solanki, S. K. *Astr. Astrophys.* **192**, 338–342 (1988).
10. Müller, R., Roudier, Th. & Hult, J. C. *Solar Phys.* **119**, 229–243 (1989).
11. Keller, C. U., Solanki, S. K., Steiner, O. & Stenflo, J. O. *Astr. Astrophys.* **233**, 583–597 (1990).
12. Dunn, R. B. & Zirker, J. B. *Solar Phys.* **33**, 281–304 (1973).
13. Title, et al. in *Physics of Magnetic Flux Ropes* (eds Russell, C. T., Priest, E. R. & Lee, L. C.), 171–179 (Am. Geophys. Un., Washington DC, 1990).
14. Müller, R. & Keil, S. L. *Solar Phys.* **87**, 243–250 (1983).
15. Schüssler, M. in *Solar Photosphere: Structure, Convection, and Magnetic Fields*, IAU Symp. 138 (ed. Stenflo, J. O.), 161–179 (Kluwer, Dordrecht, 1990).
16. Simon, G. W. & Zirker, J. B. *Solar Phys.* **35**, 331–342 (1974).
17. Beckers, J. M. & Schröter, E. H. *Solar Phys.* **4**, 142–164 (1968).
18. Zirin, H. & Wang, H. *Astrophys. J.* **385**, L27–L29 (1992).
19. Spruit, H. C. & Zwaan, C. *Solar Phys.* **70**, 207–228 (1981).
20. Knölker, M. & Schüssler, M. *Astr. Astrophys.* **202**, 275–283 (1988).

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Excited-state enhancement of optical nonlinearities in linear conjugated molecules

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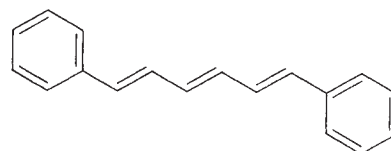
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NONLINEAR optical phenomena form the basis for all-optical devices such as optically bistable switches and nonlinear directional couplers¹. The suitability of a material for these device applications requires a large magnitude of the relevant nonlinear effect (in this case, the third-order optical susceptibility $\chi^{(3)}$, which is related to the intensity-dependent refractive index) and a small signal attenuation arising from the linear optical absorption. Conjugated organic molecules and polymers are of particular interest in this context: the delocalized π -electron systems of these materials give rise to relatively large values of $\chi^{(3)}$, with extremely fast response times, in wavelength regimes where there is minimal background absorption. Previous theoretical studies² suggested a new enhancement mechanism for the nonlinear optical processes in these materials through population of the electronic excited states. Here we show that by optically exciting a linear conjugated molecule at one wavelength into an electronic excited state for a sufficient length of time to perform the nonlinear optical measurement, the value of $\chi^{(3)}$ can be enhanced by more than two orders of magnitude without increasing optical absorption at the probe wavelength.

An electronic nonlinear optical process is said to be non-resonant when the wavelengths involved are far from any absorbing electronic transitions. Resonant processes generally have much larger nonlinear susceptibilities, but are slower because real electronic excitations occur; they also involve considerable absorptive loss of the optical beam. Nonresonant processes, as they involve only virtual electronic excitations, are essentially instantaneous, and avoid attenuation of the optical signal. The third-order nonlinear optical susceptibility is denoted $\chi^{(3)}(-\omega_4; \omega_1, \omega_2, \omega_3)$, where $\omega_4 = (\omega_1 + \omega_2 + \omega_3)$ is the frequency of the output light in response to light input at frequencies ω_1 , ω_2 and ω_3 . One standard method for measurement of $\chi^{(3)}(-\omega; \omega, \omega, -\omega)$ (where the negative sign of the final frequency argument indicates that the complex conjugate of the electric field is involved in the process) is degenerate four-wave mixing (DFWM), in which two optical beams form a phase or intensity grating that modulates the refractive index of a material and a third beam scatters off the grating in an entirely new direction³.

We have previously presented a theoretical enhancement mechanism for nonlinear optical processes originating from real population of electronic excited states in conjugated linear chains². Compared with the ground state^{4,5}, the calculated non-resonant third-order optical susceptibility $\chi^{(3)}(-\omega_4; \omega_1, \omega_2, \omega_3)$ of π -conjugated linear chain molecules can be enhanced by orders of magnitude, or even change sign, when the first (S_1) or second (S_2) electronic excited state is optically pumped and then populated for times long enough to perform nonresonant measurements of $\chi^{(3)}(-\omega_4; \omega_1, \omega_2, \omega_3)$ at frequencies different from the resonant pump frequency.

Here we report the experimental observation of excited state enhancement of the DFWM susceptibility $\chi^{(3)}(-\omega; \omega, \omega, -\omega)$ of a linear conjugated molecule, diphenylhexatriene (DPH), when the first one-photon allowed π -electron excited state is populated for nanoseconds and then probed nonresonantly through picosecond DFWM.

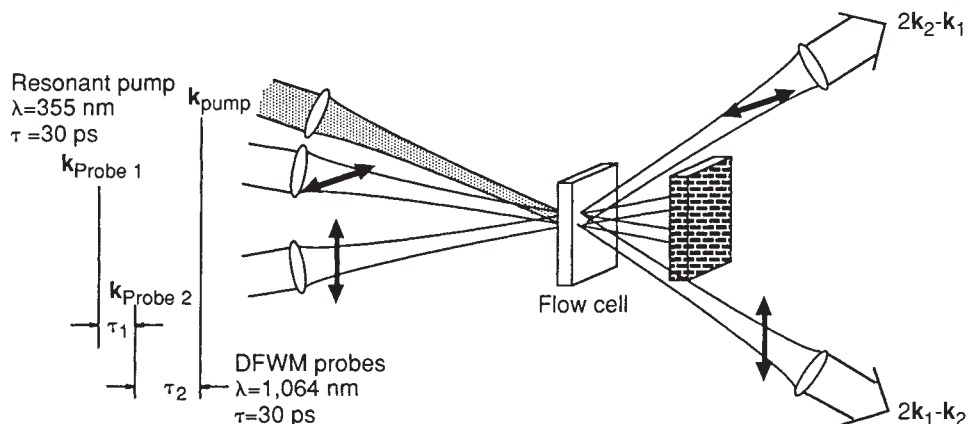


diphenylhexatriene

DPH shows saturable absorption from the $S_0(1^1A_g)$ ground state to the $S_2(1^1B_u)$ excited state centred near 350 nm, with an excited state lifetime of several nanoseconds^{6,7}, and is transparent at wavelengths from 400 nm to greater than 2,000 nm. Excitation to the S_2 state is known to lead additionally to population of the $S_1(2^1A_g)$ state, which lies at a slightly lower energy⁶. The precise assignment of excited-state population densities is irrelevant here; therefore, we refer to the excited-state population distribution hereafter simply as the S_2 state, for clarity. Fresh samples of DPH were dissolved in solutions of anhydrous dioxane, in concentrations of $1\text{--}10 \times 10^{-3}$ M, and kept isolated from atmospheric contamination.

The DFWM experiment (Fig. 1) is done using two orthogonally polarized, 1,064-nm probe beams and a 355-nm pump beam produced by a 30-ps pulsewidth, mode-locked Nd:YAG laser. The three pulsed beams, for which the relative time delays can be variably adjusted, are focused in coincidence on a thin quartz cell through which the sample solutions flow. Coherent interference of the two probes in the sample produces a refractive index grating, and the diffracted intensity of each probe from this grating, proportional to the square of the third-order optical susceptibility $\chi^{(3)}(-\omega; \omega, \omega, -\omega)$, is detected. In this DFWM arrangement there are therefore only two incident beams, rather than three³. The unique feature of our experimental configuration is that by introducing an intense optical pump beam tuned to the first electronic absorption band of the

FIG. 1 Forward-scattering DFWM experiment. The k_1 probe is horizontally polarized, the k_2 probe is vertically polarized, and the phase-matched signal in the $2k_2 - k_1$ direction is detected by an infrared active photomultiplier tube following a system of apertures and a horizontally oriented analysing polarizer.



material, we force a large fraction of the molecules in the sample to occupy the first optical excited state.

An increase as large as a factor of 100 in the completely nonresonant 1,064-nm DFWM signal from the highest DPH concentrations is observed when the 355-nm pump beam saturates the S_2 absorption as compared to when the pump beam is turned off. When no pump beam is present, the ground-state molecular susceptibility $\gamma^{S_0}(-\omega; \omega, \omega, -\omega)$ of DPH and the dioxane solvent susceptibility $\gamma^D(-\omega; \omega, \omega, -\omega)$ contribute to the net observed macroscopic susceptibility

$$\chi^{(3)}(-\omega; \omega, \omega, -\omega) = (f^\omega)^4 [N_D \gamma^D(-\omega; \omega, \omega, -\omega) + N_{S_0} \gamma^{S_0}(-\omega; \omega, \omega, -\omega)]$$

where N_{S_0} and N_D are the number densities of the ground state DPH and dioxane molecules respectively, and f^ω is the local field factor. When the pump beam is present, however, the corresponding macroscopic susceptibility is given by

$$\chi^{(3)}(-\omega; \omega, \omega, -\omega) = (f^\omega)^4 [N_D \gamma^D(-\omega; \omega, \omega, -\omega) + N_{S_0} \gamma^{S_0}(-\omega; \omega, \omega, -\omega) + N_{S_2} \gamma^{S_2}(-\omega; \omega, \omega, -\omega)]$$

where N_{S_2} and γ^{S_2} are the corresponding number density and molecular susceptibility for the S_2 state. The unpumped DFWM signal is observed to be independent of concentration, demonstrating that the ground-state $\gamma^{S_0}(-\omega; \omega, \omega, -\omega)$ for DPH is smaller than the experimental resolution of $\pm 50 \times 10^{-36}$ e.s.u. The pumped DFWM signal, however, increases strongly with increased DPH concentration, showing that the excited state $\gamma^{S_2}(-\omega; \omega, \omega, -\omega)$ is more than two orders of magnitude larger than $\gamma^{S_0}(-\omega; \omega, \omega, -\omega)$. The measured values of $\chi^{(3)}(-\omega; \omega, \omega, -\omega)$ are linearly dependent on the concentration of the solution, as expected, and yield a value for $\gamma^{S_2}(-\omega; \omega, \omega, -\omega)$ of $12,000 \pm 1,700 \times 10^{-36}$ e.s.u. On population of the excited state, the $\gamma(-\omega; \omega, \omega, -\omega)$ of DPH at a non-resonant wavelength is greatly enhanced without introducing any optical loss.

In all measurements, the DFWM signal is non-zero only when the probe pulses temporally overlap in the sample (Fig. 2). As

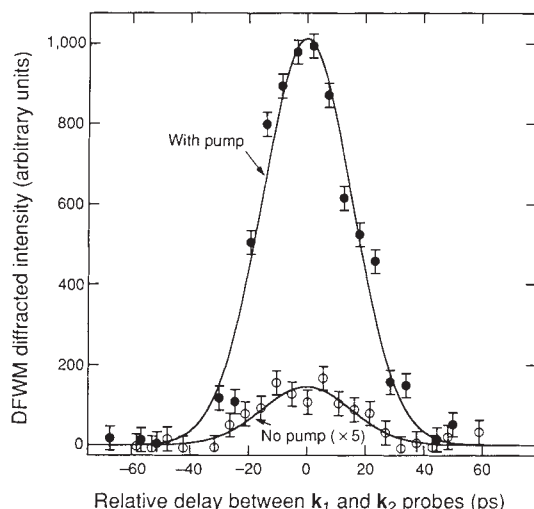


FIG. 2 Typical DFWM signal of DPH in dioxane with pump on and pump off, as a function of time delay between the two 1,064-nm probe beams. The pump delay is set so that the 355-nm pump pulse precedes the probes by 100 ps and the relative delay between the probe pulses is varied. The unpumped data, which has been multiplied by a factor of 5 for clarity, corresponds to the background signal from the dioxane solvent, because the ground-state contribution from DPH is smaller than the detection resolution of the experiment. An enhancement of a factor of 37 in intensity is shown here for a concentration of 3×10^{-3} M.

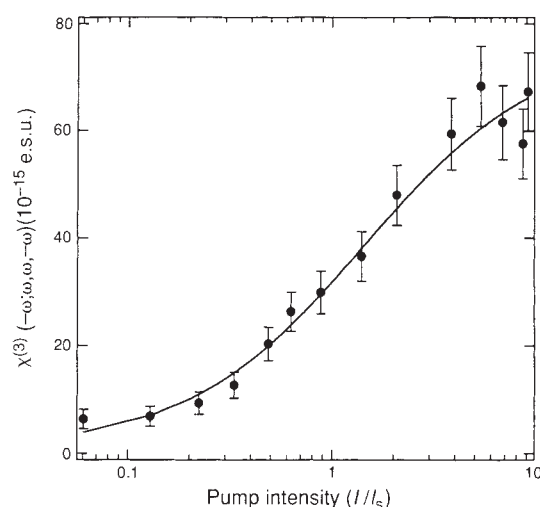


FIG. 3 Excited-state DFWM signal of DPH in dioxane as a function of pump beam intensity, demonstrating saturation. The relative delay between the probe pulses is set to zero, and the pump precedes the probes by 100 ps. At low intensity, we observe the background contribution from the dioxane solvent; at high intensity, the excited-state DPH contribution dominates.

the relative delay between the two probe pulses is adjusted, the DFWM intensity follows the expected autocorrelation of the two 30-ps pulses. Moreover, when the relative delay between the probe pulses is set to zero and the pump delay is varied, the DFWM signal shows a fast rise time followed by a nanosecond timescale decay in agreement with the probe pulse-widths and the lifetime of the S_2 state. This observed asymmetry with respect to pump pulse timing illustrates that before the pump pulse arrives, only the dioxane and DPH ground-state susceptibilities contribute, whereas at times after the pump pulse, the DPH excited-state susceptibility is detected as it decays along with the S_2 population. Additionally, the enhancement of the DFWM signal demonstrates a dependence on pump beam intensity (Fig. 3) that is in good agreement with intensity saturation of the excited-state population as found in separate saturable absorption measurements at 355 nm. Furthermore, we have done separate transient absorption experiments at 1,064 nm with excitation at 355 nm and found the excited-state absorption to be negligible.

We have observed that the DFWM signal is independent of the polarization of the pump beam, indicating that there is no coherent coupling between the pump and probes. Thus, the sole purpose of the pump beam is to create the S_2 excited-state population. The orthogonal probe polarizations ensure that the experimental DFWM signal is due entirely to the electronic third-order susceptibility of the excited state, and not due to a thermal grating. Finally, the DFWM signal is in all cases observed to have the expected cubic dependence on the probe intensities.

The enhancement mechanism demonstrated here can be generalized to second-order and other third-order nonlinear optical processes, and to other material structures, compositions and phases. The experimental observations reported here to demonstrate the principle were done on a prototype molecule with a small ground-state $\gamma^{S_0}(-\omega; \omega, \omega, -\omega)$. Furthermore, the measurements were made in solutions in which the small number density of excited state molecules with large optical nonlinearity results in a much smaller $\chi^{(3)}(-\omega; \omega, \omega, -\omega)$ than would be observed in a pure, single substance. We are currently working on pure polymer thin films where typical nonresonant ground-state values of $\chi^{(3)}(-\omega; \omega, \omega, -\omega)$ of $\sim 10^{-11}$ – 10^{-10} e.s.u. are expected to be enhanced by several orders of magnitude, possibly leading to figures of merit sufficient for practical photonics devices. □

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1. Stegeman, G. I. & Stolen, R. H. *J. opt. Soc. Am.* **B6**, 652-662 (1989).
2. Zhou, Q. L., Heflin, J. R., Wong, K. Y., Zamani-Khamiri, O. & Garito, A. F. *Phys. Rev.* **A43**, 1673-1676 (1991).
3. Kobayashi, T., Terasake, A., Hattori, T. & Kurokawa, K. *Appl. Phys.* **B47**, 107-125 (1988).
4. Heflin, J. R., Cai, Y. M. & Garito, A. F. *J. opt. Soc. Am.* **B8**, 2132-2147 (1991).
5. Heflin, J. R., Wong, K. Y., Zamani-Khamiri, O. & Garito, A. F. *Phys. Rev.* **B38**, 1573-1576 (1988).
6. Kohler, B. E. & Spiglanin, T. A. *J. chem. Phys.* **82**, 2939-2941 (1985).
7. Cehelnik, E. D., Cundall, R. B., Lockwood, J. R. & Palmer, T. F. *J. phys. Chem.* **79**, 1369-1376 (1975).

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Irregular glacial interstadials recorded in a new Greenland ice core

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THE Greenland ice sheet offers the most favourable conditions in the Northern Hemisphere for obtaining high-resolution continuous time series of climate-related parameters. Profiles of $^{18}\text{O}/^{16}\text{O}$ ratio along three previous deep Greenland ice cores¹⁻³ seemed to reveal irregular but well-defined episodes of relatively mild climate conditions (interstadials) during the mid and late parts of the last glaciation, but there has been some doubt as to whether the shifts in oxygen isotope ratio were genuine representations of changes in climate, rather than artefacts due to disturbed stratification. Here we present results from a new deep ice core drilled at the summit of the Greenland ice sheet, where the depositional environment and the flow pattern of the ice are close to ideal for core recovery and analysis. The results reproduce the previous findings to such a degree that the existence of the interstadial episodes can no longer be in doubt. According to a preliminary timescale based on stratigraphic studies, the interstadials lasted from 500 to 2,000 years, and their irregular occurrence suggests complexity in the behaviour of the North Atlantic ocean circulation.

Three deep Greenland ice cores, drilled from surface to bedrock at Camp Century, Dye 3 and Renland (Fig. 1) in 1966, 1981 and 1987, respectively, reach the ice deposited during the last glaciation¹⁻³. This conclusion is based on the occurrence of very low $\delta^{18}\text{O}$ values ($\delta^{18}\text{O}$ is the relative deviation of the ^{18}O concentration from that in the standard mean ocean water) in the deeper parts of the cores, and on evidence that $\delta^{18}\text{O}$ in polar snow and ice depends mainly on the temperature of formation of the precipitation^{4,5}. In the mid- and late glacial parts of the cores, $\delta^{18}\text{O}$ is alternately very low and intermediate (Fig. 2), corresponding to abrupt shifts between two apparently quasi-stationary climate stages⁶⁻⁸. Similar features appear in both marine^{9,10} and other terrestrial records from the North Atlantic region^{11,12}, but only weakly in Antarctic ice cores^{1,5}, if at all, which points to circulation changes in the North Atlantic Ocean as the driving force⁶.

Nevertheless, there has still been doubt about the climatic interpretation of the δ shifts. Intermittent occurrence of isotopically light surface meltwater in the North Atlantic Ocean was suggested as an alternative explanation of the low $\delta^{18}\text{O}$ values

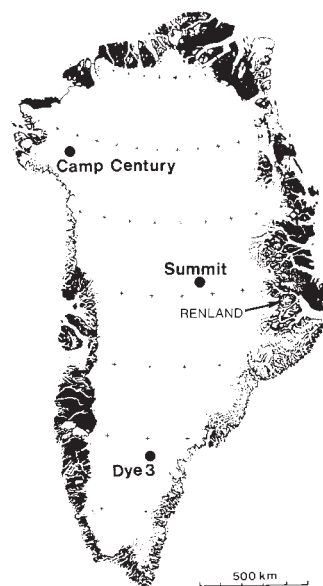


FIG. 1 Greenland with the four drill sites mentioned in the text.

during the last cold period (the Younger Dryas)¹³, but deuterium excess data ($d = \delta\text{D} - 8\delta^{18}\text{O}$) excluded this possibility⁸. Most recently, the meltwater peaks have been shown to coincide with the high- δ rather than the low- δ parts of the Dye 3 record (see Fig. 3c and d in ref. 14). Another suggested cause of the δ shifts at greater depths was disturbed stratification of the deeper part of the ice, as this ice has travelled a long way over a hilly bedrock¹⁵, or, in the case of Renland, is situated only a few metres above the bedrock³.

The Summit location on the top of the ice sheet (Fig. 1) is an almost ideal depositional environment in which to recover an ice core: the flow pattern is simple, because there is no horizontal ice movement at present, and little in the past, when the ice divide might have been slightly displaced from its present position. Furthermore, the Summit surface temperature seldom rises above the freezing point, in contrast to Dye 3 where summer melting often causes post-depositional changes in the firn, for example absorption of additional soluble gases from the atmosphere. Summit was therefore chosen as the target of a new deep core drilling under the multinational European Greenland Ice-core Project (GRIP) 1990-1992. By the end of the second field season in 1991, the drill reached a depth of 2,321 m, where the ice is ~40,000 years old.

The timescale shown in Table 1 was established by stratigraphic methods. Back to 8,600 yr BP (before present) the timescale rests on identification of reference horizons in the form of acid volcanic fall-out dated in the Dye 3 core by counting annual $\delta^{18}\text{O}$ variations downwards from the surface¹⁶. Before 8,600 yr BP, the dating was accomplished by a multi-parameter method that identifies the seasonal variations of the concentrations of Ca^{2+} , microparticles, NH_4^+ and nitrate. This is extremely laborious, of course, and the resulting timescale (Table 1) is preliminary. Details will be reported elsewhere.

Figure 2 shows continuous $\delta^{18}\text{O}$ profiles along the four Greenland ice cores, plotted on linear depth scales that span the depth intervals listed in Table 2. The Summit depth scale is shown at the outer left along with the stratigraphically derived Summit timescale. The heavy and thin vertical lines indicate δ levels characteristic of low and intermediate δ , respectively.

The close correlation between the Summit and Dye 3 records rules out disturbed stratigraphy as a potential cause for the two δ levels, which must therefore be of climatic significance. Periods of relatively high δ reflect mild interstadials (IS), detectable in all four records (except for IS 3 at Camp Century). The absence

of IS 3 at Camp Century, as well as the 'extra' interstadial just below IS 1e, may be ascribed to the less advanced ice-core processing techniques applied to the cores 26 years ago. IS 1 is identical with the Bølling and Allerød mild periods in Europe, but it is not clear how to separate them by an 'Older Dryas' cold spell.

Smoothed versions of the records have minima between IS 2 and IS 5, around 25,000 yr BP. The temperature minimum thus occurred before the often quoted time of maximum glacier extent (18,000 yr BP¹⁶, corresponding to ~21,500 calendar years BP¹⁸). This suggests a time lag of more than 3,000 yr for the build-up of great ice sheets, in essential agreement with a climate model that simulates the transient response of the cryosphere to astronomical forcing of climate¹⁹.

The right-hand side of Table 2 lists the mean δ values, δ_p , δ_m and δ_c (for present, mild-glacial and cold-glacial stages, respectively), and the differences $\Delta_{mc} = \delta_m - \delta_c$, $\Delta_{pm} = \delta_p - \delta_m$ and $\Delta_{pc} = \delta_p - \delta_c$. As regards the three deep cores, the Δ_{mc} values are similar, but the Camp Century values of both Δ_{pm} and Δ_{pc} are significantly higher than those found at Summit and Dye 3. This may be explained by the changing altitude of the deposition area at Camp Century during the glacial to post-glacial transition²⁰, and the termination of the shadow effect of the shrinking Laurentide ice-sheet. The conditions at the small isolated Renland ice cap are more complicated³, but all of the interstadials are easily recognized.

The present geographical relationship between $\delta^{18}\text{O}$ (in ‰) and temperature (T in °C)⁸

$$\delta^{18}\text{O} = 0.67T - 13.7$$

seems valid also for substantial temporal changes^{4,21}, and

TABLE 1 Depth/time for Summit ice core

Depth (m)	Time (yr)		Event
58.85	1,816	AD	V Tambora
67.60	1,783	AD	V Laki
110.95	1,601	AD	V Unknown
139.40	1,477 ± 2	AD	V Unknown
187.25	1,259 ± 2	AD	V Unknown
203.80	1,179	AD	V Katla
256.15	934 ± 3	AD	V Eldgá
455.35	2,039 ± 5	BP	V Unknown
736.45	3,636 ± 7	BP	V Thera
802.75	4,040 ± 10	BP	V Unknown
1,334.50	8,210 ± 30	BP	C $\delta^{18}\text{O}$ minimum
1,380.50	8,600 ± 50	BP	V Unknown
1,623.60	11,550 ± 70	BP	C Transition to Pre-boreal
1,661.55	12,700 ± 100	BP	C Onset of Younger Dryas
1,753.40	14,450 ± 200	BP	C Onset of the Bølling interstadial
1,766.00	15,000 ± 250	BP	
1,884.00	20,000 ± 800	BP	
2,006.00	25,000 ± 1,000	BP	
2,113.00	30,000 ± 1,300	BP	
2,225.00	35,000 ± 1,600	BP	
2,321.40	40,000 ± 2,000	BP	End of 1991 core

Depth against time-of-formation relationship for strata in the Summit ice core, of which those marked by V contain acid fall-out from discrete volcanic eruptions back to 8,600 yr BP (before AD 1990). The strata marked by C indicate notable climatic events from 8,210 to 14,450 yr BP detectable in all of the ice cores listed in Table 2. The $\pm$ values are estimated error limits. No error has been put on the time of historically well dated volcanic events.

FIG. 2 Continuous $\delta^{18}\text{O}$ profiles along sections of four Greenland ice cores from Summit (Central), Dye 3 (Southeast), Camp Century (Northwest) and Renland (East Greenland), spanning nearly the same time interval. The four records are all plotted on linear depth scales, of which only the Summit depth scale is shown to the left along with a Summit timescale. The heavy and thin vertical lines indicate estimated δ levels characteristic of late-glacial cold and mild stages respectively. The figures close to these lines define a suggested numbering of significant mid- and late-glacial interstadials.

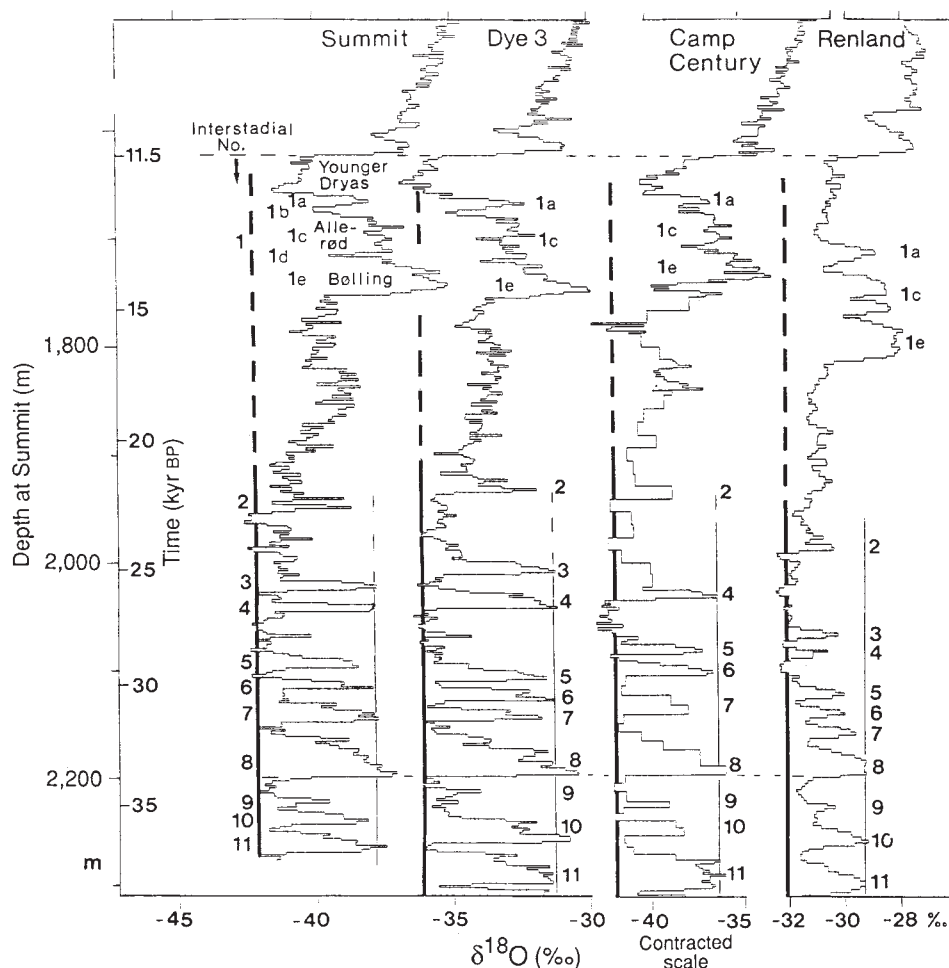


TABLE 2 Ice core data

Drill site	Location	Altitude above sea level (m)	Mean air temperature (°C)	Accumulation rate (cm ice per yr)	Ice-sheet thickness (m)	Ice-core length (m)	Increment shown in Fig. 2 (m)	δ_o (‰)	δ_m (‰)	δ_c (‰)	Δ_{mc} (‰)	Δ_{pm} (‰)	Δ_{pc} (‰)
Summit	72° 34' N 37° 37' W	3,230	-32	23	3,030	2,321	1,500-2,281	-35.3	-37.7	-41.9	4.2	2.4	6.6
Dye 3	56° 11' N 43° 50' W	2,490	-20	56	2,037	2,037	1,762-1,919	-27.9	-31.2	-36.1	4.9	3.3	8.2
Camp Century	77° 11' N 61° 07' W	1,890	-24	38	1,390	1,390	1,149-1,248	-29.0	-36.4	-41.9	5.5	7.4	12.9
Renland	71° 18' N 26° 44' W	2,350	-18	50	325	325	305.4-313.0	-27.1	-29.3	-32.1	2.8	2.2	5.0

indicates that the cold glacial stages (Fig. 2) were $\sim 7^\circ\text{C}$ colder than the mild ones, and $12\text{--}13^\circ\text{C}$ colder than at present (when corrected for the higher glacial δ value of the oceans²²), in agreement with combined ice and heat-flow modelling²¹. Temperature fluctuations of this size in Greenland must have been associated with significant environmental changes in the entire North Atlantic region, particularly in Europe, as seen for example in the botanical evidence of 'swift climatic changes' during the last glaciation¹².

Figure 2 further shows that the duration of the interstadials ranges from about 500 to 2,000 yr. They begin abruptly, perhaps within a few decades^{14,23} (the estimated 50-year duration of the Younger Dryas to Pre-boreal transition in ref. 23 has been confirmed by new annual layer counting on the Summit core), but they terminate gradually or in a stepwise fashion. Furthermore, the interstadials apparently occur at irregular intervals. The driving force is probably changing intensity and/or direction of the North Atlantic Current, associated with changing sea ice cover^{8,22} and deep water formation^{10,14}, but the interstadials have not been satisfactorily modelled. Even the mechanism behind the most thoroughly studied interstadial (IS 1) is still debated^{24,25}.

If the interstadials turn out to reflect a randomness in the general pattern of atmosphere-ocean circulation, comprising two or more possible flow schemes for a given set of primary parameters, climate modellers will have to reconsider the feasibility of predicting natural climate change. The glacial interstadials were indeed linked to changing circulation in an ocean partly covered by sea ice, but it should be borne in mind that within the last millennium, the well documented medieval warmth in Europe was gradually replaced by the 'little ice age', when Iceland was frequently surrounded by sea ice, and the succeeding warming culminated in an abrupt temperature increase in the 1920s, too abrupt to be explained by the increasing greenhouse effect. This oscillation had smaller amplitudes than its glacial predecessors, but a similar sequence of events (gradual cooling followed by abrupt warming). □

24. Veum, T., Jansen, T., Arnold, M., Beyer, I. & Duplessy, J. C. *Nature* **356**, 783-785 (1992).
25. Zahn, R. *Nature* **356**, 744-746 (1992).

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Reconciling particulate organic carbon flux and sediment community oxygen consumption in the deep North Pacific

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THE mineralization of organic carbon in the deep ocean has been considered an enigma in the North Pacific. Comparisons of the supply of particulate organic carbon (POC) with estimates of its mineralization to CO_2 by the sediment community have indicated that the supply of POC sinking into the benthic boundary layer may be as much as 97% short of meeting the organic carbon demand of the sediment community^{1,2}. These previous findings were based on short-time-series measurements (<14 days) conducted *in situ* with sediment traps and benthic respirometers. We report here a comparison of long-time-series measurements (2.3 years) of POC flux into the benthic boundary layer and concurrent, seasonal measurements of sediment community oxygen consumption. We chose a single abyssal station (4,100 m depth) in a region of the eastern North Pacific where there is a strong seasonal fluctuation in primary production, and where previous studies^{1,2} showed the supply of POC to be as much as an order of magnitude lower than the demand by the sediment community. Our measurements, using the same methods, show agreement to within 15% between organic carbon supply and demand. This reconciliation is attributable to the inclusion of previously undetected episodic inputs of POC into the benthic boundary layer. These episodic inputs are critical to sustaining the sediment community at this station, and are probably important in other deep-sea environments where seasonal fluctuations in primary production are prominent in surface waters.

Short-time-series measurements of POC flux into the benthic boundary layer (BBL) and its utilization by the sediment community, as estimated from sediment community oxygen consumption (SCOC), have been made in the North Atlantic and North Pacific oceans (Table 1). POC fluxes at the Atlantic stations are sufficient to meet the demands of SCOC. But similar methods used in the Pacific have shown that there is a shortfall in the supply of POC necessary to meet the demands of the sediment community at numerous stations stretching from the continental margin off California to abyssal stations in the central gyre of the North Pacific. Several hypotheses have been suggested to account for the discrepancy in the North Pacific,

Received 26 May, accepted 24 August 1992.

- Johnsen, S. J., Dansgaard, W., Clausen, H. B. & Langway, C. C. Jr *Nature* **235**, 429-434 (1972).
- Dansgaard, W. et al. *Science* **208**, 1273-1279 (1982).
- Johnsen, S. J. et al. *Medd. Grønland Geosci.* **30**, 22 pp. (1992).
- Dansgaard, W. et al. *Nature* **255**, 24-28 (1975).
- Jouzel, J., Lorius, C., Petit, J. R., Genthon, C. & Barkov, N. I. *Nature* **329**, 403-407 (1987).
- Dansgaard, W., Johnsen, S. J., Clausen, H. B. & Langway, C. C. Jr in *Late Cenozoic Ice Ages* (ed. Turekian, K. K.) 37-56 (Yale Univ. Press, 1970).
- Oeschger, H. et al. in *Climate Processes and Climate Sensitivity* (eds Hansen, J. E. & Takahashi, T.) *Geophys. Monogr.* **29**, 299-306 (Am. Geophys. Un., Washington DC, 1984).
- Johnsen, S. J., Dansgaard, W. & White, J. W. *Tellus* **B41**, 452-468 (1989).
- Ruddiman, W. F. & McIntyre, A. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **35**, 145-214 (1981).
- Broecker, W. S., Bond, G., Klas, M., Bonani, G. & Wolff, W. *Palaeoceanography* **5**, 469-477 (1990).
- Woillard, G. M. & Mode, W. D. *Science* **215**, 159-161 (1982).
- Kolstrup, E. *Geol. Mijnbouw* **69**, 253-262 (1990).
- Fairbanks, R. G. *Nature* **342**, 637-642 (1989).
- Lehmann, S. J. & Keigwin, L. D. *Nature* **356**, 757-762 (1992).
- Overgaard, S. & Gundestrup, N. in *Greenland Ice Cores: Geophysics, Geochemistry and Environment* (eds Langway, C. C. Jr, Oeschger, H. & Dansgaard, W.) *Geophys. Monogr.* **33**, 49-56 (Am. Geophys. Un., Washington, DC, 1985).
- Hammer, C. U., Clausen, H. B. & Tauber, H. *Radiocarbon* **28**, 2A, 284-291 (1986).
- CLIMAP project members *Science* **191**, 1131-1137 (1976).
- Bard, E. *Radiocarbon* (in the press).
- Berger, A. et al. *Trans. R. Soc. Edinb. Earth Sci.* **82**, 357-369 (1990).
- Raynaud, D. & Lorius, C. *Nature* **243**, 283-284 (1973).
- Dahl-Jensen, D. & Johnsen, S. J. *Nature* **320**, 250-252 (1986).
- Shackleton, N., Imbrie, J. & Hall, M. A. *Earth planet. Sci. Lett.* **65**, 233-244 (1983).
- Dansgaard, W., Johnsen, S. J. & White, J. C. *Nature* **339**, 532-534 (1989).

Table 1 Comparison of POC flux and SCOC

Station			POC flux			SCOC			Ref.
Location	Depth (m)	Season	Altitude (m.a.b.)	Measurement period (days)	Flux ($\text{mg C m}^{-2} \text{d}^{-1}$)	Measurement period (days)	Requirement ($\text{mg C m}^{-2} \text{d}^{-1}$)	POC flux/SCOC	
North Pacific	4,400	Sp, A	100 & 600	4-7	0.2-5.4	≤ 5	7.9-14.0	0.03-0.4	1
	4,900	Sp, A	100 & 600	4-7	0.2 & 0.6	≤ 5	0.9 & 2.0	0.2 & 0.3	
	5,800	Sp, A	100 & 600	7	0.4-1.4	≤ 5	0.7-2.8	0.2-1.9	
	4,400	Sp, A	600	7-14	1.0-2.3	2-5	8.6-12.0	0.09-0.3	2
	5,800	Sp, S, A	600	7-14	0.2-0.9	2-5	0.9-2.1	0.2-1.1	
North Atlantic	3,000	Sp, S	36-118	5-15	9.3	2-5	2.2	4.2	8
	3,650	Sp, S	36-118	5-15	18.1	2-5	2.6	7.0	
	3,515	Sp	50	NA	7.7	NA	2.3	3.3	15
	4,000	Sp	NA	NA	30.8	6	8.5	3.6	
	3,650	Sp	518	16	11.5	2-5	2.6	4.4	16

Fluxes measured at abyssal depths ($\geq 3,000$ m water depth) in the North Atlantic and North Pacific. All fluxes have been normalized to one square metre of the bottom; the altitude of the POC flux measurement is specified for each study. Season when measurements were conducted: Sp, spring; S, summer; A, autumn; W, winter.

ranging from methodological problems with measuring POC flux and SCOC¹ to the existence of other carbon sources that are not sampled with conventional sediment traps^{3,4}.

To address the discrepancy between POC flux and SCOC in the North Pacific, we undertook a long-time-series study over 2.3 years (June 1989–October 1991; 852 days) to measure the sinking POC entering the BBL at a single abyssal station, roughly 220 km west of central California. In addition, seasonal measurements of SCOC were made over the same period of time as the POC flux measurements.

The surface waters that overlie the study site (station M; 4,100 m depth at 34° 50' N, 123° 00' W) are part of the California Current, and have well-developed spring plumes of chlorophyll which persist into summer^{5,6} and vary from year to year⁷. Primary production associated with these areas is roughly three times

higher in spring than in winter (T. L. Hayward, unpublished work). Station M has a BBL characterized by a slight decrease in light transmission ($\sim 1\%$) in the bottom 200 m of the water column. Temperature, dissolved oxygen and salinity profiles in the bottom 1,000 m are similar in winter, spring and autumn, with no obvious vertical discontinuities to suggest boundary limits. Mean current speeds, measured at 2, 50, 100 and 600 m above bottom (m.a.b.), range from 2.9 cm s^{-1} at 2 m.a.b. to 3.5 cm s^{-1} at 50 m.a.b., with maximum speeds $\leq 7 \text{ cm s}^{-1}$ (K.S., unpublished data). The sea floor is composed of silty clay with little topographic relief (< 60 m relief over an area of 770 km^2).

Fluxes of POC, measured with sediment traps at 600 and 50 m.a.b., showed considerable interannual variability, with increased rates in the spring and autumn (Fig. 1). The highest fluxes of POC occurred in spring and summer of 1991, at a time

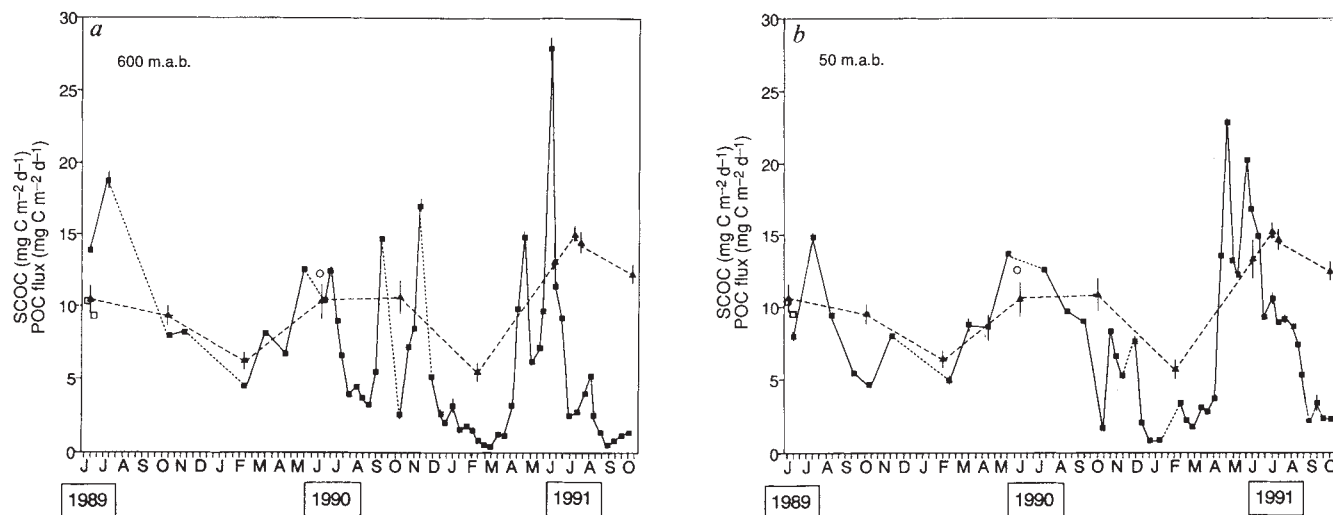


FIG. 1 Fluxes of particulate organic carbon (POC; solid line) at (a) 600 m.a.b. and (b) at 50 m.a.b. compared with sediment community oxygen consumption (SCOC; dashed line) from June 1989 to October 1991. POC analyses were done in duplicate and are presented as maximum, minimum and mean. Periods when POC collections failed are represented by dotted lines. SCOC values are mean rates presented with ± 1 standard deviation. Additional SCOC measurements made in June 1989⁴ ($\square$) and June 1990⁹ ($\circ$). METHODS. Sediment traps were placed at 50 and 600 m.a.b. on a single mooring. Each trap consisted of a steep-sided funnel with swiveled bridle and had an effective mouth opening of 0.25 m^2 (ref. 17). A baffle at the top of each funnel reduced turbulence. A sequencer, bolted to the bottom of each funnel, was capable of flushing on descent and ascent and taking 12 discrete samples at depth¹⁸, providing 10-day resolution. Before June 1990 at 600 m.a.b. and October 1990 at 50 m.a.b., sequencers with four sample positions were used and provided 30-day sampling resolution. The sediment traps were recovered, serviced and redeployed within 2 days at

four-month intervals. Current speeds measured over the last two years at 600 and 50 m.a.b. range from < 0.5 – 5.2 cm s^{-1} ($\bar{x} = 1.5 \text{ cm s}^{-1}$). Collection cups were filled before deployment with water obtained from the deployment depth, filtered through pre-combusted GF/C filters and poisoned with 3.0 mM HgCl_2 . Samples of the initial and final collection water were frozen and triplicate subsamples were analysed for dissolved organic carbon using a high-temperature catalytic oxidation method¹⁹. On recovery of the sediment traps, overlying water in each cup was sampled for DOC and 'swimmers' were removed. Samples were then frozen and later analysed in duplicate for total and inorganic carbon using a Perkin-Elmer elemental analyser and Coulometrics carbon analyser, respectively. Organic carbon was determined as the difference between total and inorganic carbon, with a correction for salt content. SCOC was measured *in situ* with a free-vehicle grab respirometer, using methods described by Smith¹. The bottom water oxygen concentration ranged from 142 to $146 \mu\text{mol l}^{-1}$ ($\bar{x} = 143 \mu\text{mol l}^{-1}$) from June 1989 to October 1991.

when the chlorophyll concentration and primary production are typically high in surface waters. Integration of the curve for POC flux over the 852-day sampling period yielded $3,867 \text{ mg C m}^{-2}$ at 600 m.a.b. and $4,024 \text{ mg C m}^{-2}$ at 50 m.a.b., or an annual flux of 1,657 and $1,724 \text{ mg C m}^{-2} \text{ yr}^{-1}$, respectively. The close agreement between these estimates (within $\sim 4\%$) suggests that inputs through horizontal advection have little effect on the POC fluxes measured between these altitudes. Localized events of bottom resuspension, may, however, account for some periods (such as spring/summer 1991) when the POC flux was greater at 50 m.a.b. than at 600 m.a.b.; these periods do not affect the long-term agreement between fluxes at 600 and 50 m.a.b.

Sediment community oxygen consumption was measured *in situ* with a free-vehicle grab respirometer¹ on seasonal cruises over the period of the sediment trap collections. Short-term (2-day) measurements of SCOC taken at 4-month intervals and converted to carbon demand, assuming a respiratory quotient of 0.85 (ref. 8), generally follow the seasonal patterns in POC flux, with increased rates between June and October and lowest rates in February (Fig. 1). SCOC was measured at 4-month intervals, rather than the contiguous 10-day intervals for the POC flux. More closely spaced SCOC measurements, taken in June, July and August 1991, showed fine-scale changes which were not evident in the more widely spaced measurements (June and October 1991). Independent measurements of SCOC made *in situ* at station M with a benthic experimental chamber in June 1989 (ref. 4) and with an *in situ* microprofiler in June 1990 (ref. 9) agreed with those reported here (Fig. 1).

Integration of the curve for SCOC over the 2.3-year sampling period yielded a carbon consumption of $4,913 \text{ mg C m}^{-2}$ or an annual rate of $2,105 \text{ mg C m}^{-2} \text{ yr}^{-1}$. If we compare the supply of organic carbon (POC flux) with the sediment community demand as estimated from SCOC, the supply measured at 600 and 50 m.a.b. meets 78.7% and 81.9% of the demand, respectively. Previous short-time-series measurements of POC flux and SCOC made using similar methods at a station 250 km southwest of station M (station F, water depth 4,400 m) showed the supply to range from 3% to 40% of the demand (Table 1) during periods in spring, summer and autumn from 1982 to 1986 (refs 1, 2). Although similar discrepancies in supply and demand do occur in the late summer and autumn months at station M, there are periods in the spring and summer when the POC flux exceeds the SCOC (Fig. 1).

Long-time-series measurements have shown closer agreement between the supply of and the demand for organic carbon than has been previously demonstrated in the eastern North Pacific, but there is still a shortfall of POC flux compared with the SCOC. One possible reason for this discrepancy is that POC measurements may underestimate the carbon flux to the BBL because of dissolution in the sediment traps. During our time-series measurements at station M, we began measuring the initial and final concentrations of dissolved organic carbon (DOC) in the collection cups of the sediment traps. The sampling of DOC in the collection cups began intermittently in February 1990 and then continuously after May 1991. The fraction of the POC flux which dissolved into DOC was highly variable and probably related to the particle composition (Fig. 2). We estimated the contribution of the dissolved fraction of POC flux over the 606-day period from February 1990 to October 1991 by connecting intermittent and continuous measurements and integrating the curve. When this dissolved fraction of organic carbon was combined with the POC flux and compared to SCOC for the same period of time, there was closer agreement between the supply of POC and the demand for organic carbon by the sediment community. An integration of POC flux combined with the dissolved fraction over the 606-day period yields $3,036 \text{ mg C m}^{-2}$ at 50 m.a.b., constituting 85% of the estimated SCOC for this same period ($3,594 \text{ mg C m}^{-2}$). Although the data set for estimating the dissolved fraction of the POC flux is

incomplete, the discrepancy between the POC flux measured at 50 m.a.b. and the SCOC is considerably less than previously reported from short-time-series measurements in the North Pacific and is now within the limits of measurement errors for these methods¹.

Our results from these long-time-series measurements suggest that the apparent discrepancies in previous short-time-series measurements of POC flux are due to episodic inputs of POC which were not adequately sampled. These episodic inputs of POC are critical to sustaining the sediment community at station M and are probably important in other abyssal areas where seasonal fluctuations in primary production are prominent in surface waters.

We propose that the previously unmeasured episodic inputs of sinking POC are also responsible for the imbalance observed on a broader spatial scale in the Pacific¹. Episodic fluxes of POC are also prevalent in the Atlantic¹⁰, however, and given similar estimates of primary productivity in the Pacific and Atlantic¹¹, the question remains why higher rates of SCOC have been reported in the Pacific¹². Berger and associates¹¹ proposed that the narrower continental shelf and increased steepness of

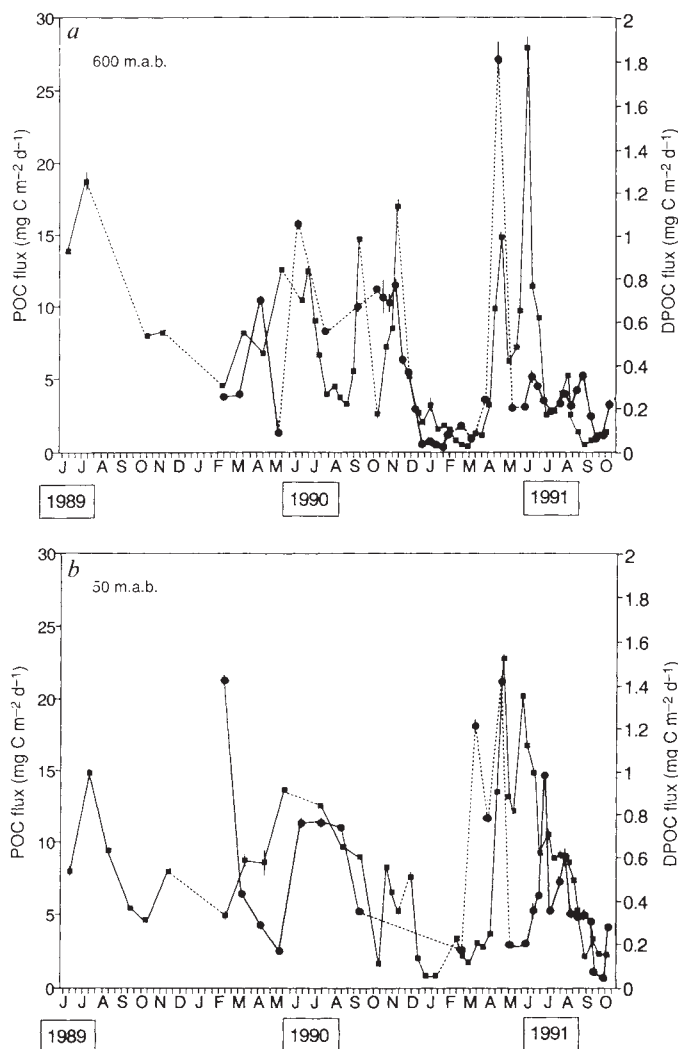


FIG. 2 Flux of particulate organic carbon (POC; ■) at (a) 600 m.a.b. and (b) 50 m.a.b. compared with the fraction of the POC that dissolved during collection (DPOC; ●). The dissolved fraction of the POC was sampled intermittently beginning in February 1990 and then continuously after May 1991. Periods when no data were collected are connected by dotted lines. The flux of the dissolved fraction was determined by subtracting the initial from the final concentration of DOC in the collection cups using methods described in Fig. 1.

the slope in the eastern North Pacific resulted in more effective transfer of coastal production to the deep-sea floor and thus increased SCOC. Gardner and Richardson¹³ argued that the high eddy kinetic energy at abyssal depths in the western North Atlantic increased bottom resuspension, resulting in (1) longer residence time of POC in the water column, (2) greater utilization of POC by plankton in the near-bottom water of the BBL and (3) lower mineralization rates in the sediment. This suggested intermediate step in the mineralization of organic carbon by the BBL plankton has been effectively examined only in the North Pacific, where carbon utilization (oxygen consumption) rates of the plankton community are low compared with those measured in the sediments¹⁴. If the BBL plankton are important consumers

of organic carbon in the North Atlantic, then the influence of episodic fluxes would be less apparent in the sediment community alone. In the North Pacific, however, where the sediment community is the most important consumer of carbon, the influence of episodic changes in POC flux on SCOC should be more prevalent and discrepancies between these rates more acute.

Our results stress the importance of long-time-series measurements, and the need to resolve episodic events and the activity of primary consumer groups in the BBL of the Atlantic and Pacific Oceans, if we are to elucidate variations of the carbon cycle in the deep ocean. □

Received 1 June; accepted 4 August 1992.

- Smith, K. L. Jr *Limnol. Oceanogr.* **32**, 201–220 (1987).
- Smith, K. L. Jr *Deep Sea Res.* **36**, 1111–1119 (1989).
- Smith, K. L. Jr., Alexandrou, D. & Edelman, J. L. *Deep Sea Res.* **36**, 1427–1441 (1989).
- Jahnke, R. A., Reimers, C. E. & Craven, D. B. *Nature* **348**, 50–54 (1990).
- Smith, R. C., Zhang, X. & Michaelson, J. *J. geophys. Res.* **93**, 10863–10882 (1988).
- Michaelson, J., Zhang, X. & Smith, R. C. *J. geophys. Res.* **93**, 10883–10896 (1988).
- Pelaez, J. & McGowan, J. A. *Limnol. Oceanogr.* **31**, 927–950 (1986).
- Smith, K. L. Jr *Mar. Biol.* **47**, 337–347 (1978).
- Reimers, C. E., Jahnke, R. A. & McCorkle, D. C. *Global biogeochem. Cycles* **6**, 199–224 (1992).
- Deuser, W. G. *Deep Sea Res.* **33**, 225–246 (1986).
- Berger, W. H., Fischer, K., Lai, C. & Wu, G. *Undersea Res. Symp.*, 88–1 (ed. Agegian, C.) 131–176 (National Oceanic and Atmospheric Administration, Silver Springs, 1988).

- Smith, K. L. Jr & Hinga, K. R. *The Sea*, Vol. 8 (ed. Rowe, G. T.) 331–370 (Wiley, New York, 1983).
- Gardner, W. D. & Richardson, M. J. *Deep-Sea Food Chains and the Global Carbon Cycle* (eds Rowe, G. T. & Pariente, V.) 339–364 (Kluwer, Dordrecht, 1992).
- Smith, K. L. Jr *Limnol. Oceanogr.* **37**, 1034–1056 (1992).
- Hinga, K. R., Sieburth, J. McN. & Heath, G. R. *J. mar. Res.* **37**, 557–579 (1979).
- Rowe, G. T. & Gardner, W. D. *J. mar. Res.* **37**, 581–600 (1979).
- Guland, K. W., Franks, R. P., Landing, W. M. & Soutar, A. *Earth planet. Sci. Lett.* **53**, 400–408 (1981).
- Honjo, S. & Doherty, K. W. *Deep Sea Res.* **35**, 133–149 (1988).
- Williams, P. M., Bauer, J. E., Robertson, K. J., Wolgast, D. M. & Ocellini, M. *Mar. Chem.* (in the press).

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Lack of nitrogen cycling in the Atacama Desert

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MESQUITE (*Prosopis*) trees growing in the rainless region of the Atacama Desert produce leaves that abscise and accumulate on a concrete-like carbonate surface, often attaining litter depths of 45 cm. The virtual lack of surface moisture inhibits leaf decomposition, and prevents cycling of nitrogen, the mineral most often limiting plant growth. Leaves in the midpoint of a litter profile were aged to pre-bomb dates (older than 1950) and had both high nitrogen concentrations and a carbon to nitrogen ratio comparable to that of live leaves. The thick carbonate layer prevents root growth into the litter. *Prosopis* appear to persist by having roots that fix nitrogen in moist subsurface layers and by extracting water and other nutrients from ground water, allowing plants to persist in an ecosystem in which there is no nitrogen cycling.

The Pampa del Tamagual is a closed basin (salar) in the Atacama Desert, east of Iquique, Chile (between 19 and 21° S). This region receives essentially no rainfall¹, yet supports a forest of two *Prosopis* species². *Prosopis tamarugo* is native to the area; *Prosopis alba* was introduced from Argentina in pre-Columbian times². Trees of both species obtain their moisture from groundwater at depths of 6–8 m or more^{3–5}. This groundwater originates in the higher Andes with a replacement time of hundreds to thousands of years^{1,5}.

The salar surfaces are covered by a 20–60-cm-thick, concrete-like layer of carbonates. The lack of rainfall and low humidity levels¹ make it likely that litter decomposition should occur at

extremely slow rates. Thus, although minerals are extracted from the soil by roots and transformed into biomass, subsequent litter from dead above-ground tissues would not be incorporated back into soil for recycling by plants. Here we explore this possibility and its ramifications for *Prosopis alba* and *P. tamarugo*.

Both *Prosopis* species have an umbrella-like canopy with the outer branches reaching to the substrate surface. This structure protects the litter layer from wind dispersion. Under normal cultural practices, these outer branches are removed so that grazing animals can forage and seek shelter under the trees. But unpruned trees commonly occur as isolated individuals in remote sites.

In December 1990, we located isolated *Prosopis alba* in the centre of a large salar free from the influence of grazing animals. There was considerable accumulation of leaf litter under the trees reaching depths of 45 cm. Litter samples were collected at 5-cm depth intervals from two sample excavations. The litter layer terminated on the top of the impenetrable 30–40-cm-thick crystalline salt pan of carbonates. The litter samples were then analysed for carbon and nitrogen contents using an elemental analyser (model 2400, Perkin-Elmer) and for magnesium, potassium, and sodium contents using an arc spectrometer system⁶. We also collected both live and dead leaves attached to the tree for parallel analyses.

The nitrogen contents of the live leaves were relatively high, as is characteristic of nitrogen-fixing plants (Fig. 1). The leaf litter was similarly high in nitrogen and these values did not significantly change with depth ($r = 0.44$, $n = 10$, $p = 0.239$). The nitrogen content of dead leaves in the litter was not significantly different from that of live leaves (Student's $t = 1.271$, $P = 0.236$). The carbon to nitrogen ratio, an index of carbon loss through decomposition, varied between 10 and 12 (Fig. 1). Visually there were no discernable structural differences between the leaves at the top and bottom of the litter layer. Thus, there was little indication that any of the material from the nitrogen-rich leaf litter was being returned to the soil, especially given both the thick carbonate layer preventing root growth into the litter and lack of rainfall to induce decomposition activity.

How old was the litter and could it possibly represent all of the litter deposited during the lifetime of these trees? Litter samples were analysed for ¹⁴C content by the University of Arizona Radiocarbon Laboratory⁷. These results indicated that all litter samples at depths 20 cm or more below the litter surface

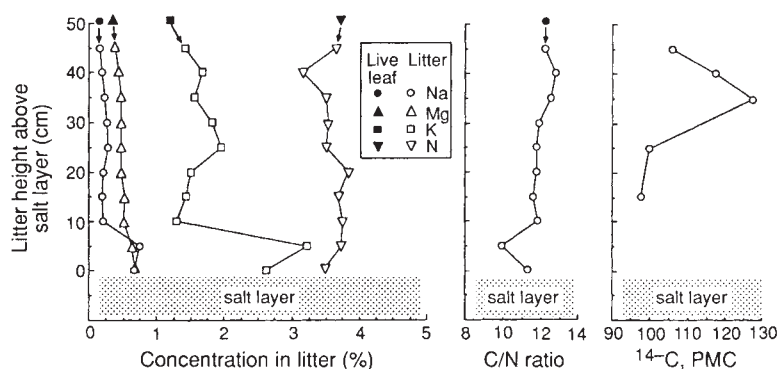


FIG. 1 Magnesium, nitrogen, potassium, sodium, carbon to nitrogen ratio and ^{14}C contents (per cent of modern carbon) of litter from different depths underneath *Prosopis alba* in the Pampa del Tamarugal of Northern Chile.

were pre-1950 (not modern carbon) (Fig. 1). The ^{14}C peak associated with above-ground nuclear testing in the 1950s and early 1960s and its decay following the Test Ban Treaty are visible in these data. Roughly 45% of the existing litter had been produced during the past 40 years and the other 55% before 1950. By linear extrapolation, the oldest litter was a minimum of 89 years old. Independently, we can estimate an age for the deepest litter, knowing productivity rates and accumulation depth. Productivity studies with mature *Prosopis* in the Pampa del Tamarugal indicate that trees produce $0.7\text{--}1.0\text{ kg m}^{-2}$ leaf litter per year⁸. The accumulated litter profile averaged $85.58 \pm 16.38\text{ kg m}^{-2}$ (mean $\pm$ s.d.), indicating an accumulation of 86–122 years in a 45-cm litter layer, if decomposition has not occurred and we simply divide the total litter accumulation by a constant litter production rate. The latter estimate is likely to be a minimum age for the deepest litter layers, because the litter accumulation rate should increase as trees mature.

Although there is apparently no return of minerals from above-ground biomass to the soil, this is not the case for underground root tissues. *Prosopis* growing in this soil produce a dimorphic root system, with a tap root that reaches the water table at 8–12 m depth and a second dense mesh of fine roots at one or more depths between 50 and 200 cm below the salt crust³. Root hydraulic lift results in a moist layer in the upper soil regions immediately adjacent to the major roots^{3,4} and root nodules capable of nitrogen fixation were abundant in these wetted, upper soil layers.

Possible sources of plant tissue nitrogen were determined by nitrogen isotope analyses of plant and soil materials⁹. Nitrogen isotope ratios ($\delta^{15}\text{N}$) of *Prosopis* leaves were measured by combusting plant material with Cu, CuO, CaO, and silver foil under vacuum at 850°C ^{10,11}, isolating and purifying the diatomic nitrogen, and measuring its nitrogen isotope ratio on a mass spectrometer (delta S, Finnigan MAT).

The current leaf $\delta^{15}\text{N}$ values averaged 0.3‰ and 0.4‰ for *P. alba* and *P. tamarugo*, respectively. These values are virtually identical to that previously reported for *P. glandulosa* in North America and are typical of plants exclusively obtaining their nitrogen through nitrogen-fixation⁹. A second analysis was conducted using *Pluchea absinthioides* (Asteraceae) and *Distichlis thalassica* (Poaceae), both non-nitrogen-fixing plants occurring occasionally in *Prosopis* stands. *Pluchea* leaf $\delta^{15}\text{N}$ values averaged 7.2‰, whereas those of *Distichlis* averaged 6.7‰. Both values serve as a reference for the $\delta^{15}\text{N}$ value of mineral N in the ecosystem, derived either from soils or from groundwater sources. Soils beneath the *Prosopis* canopies at the same depths as nodules had $\delta^{15}\text{N}$ values that averaged 10.2‰, consistent with an expected isotopic fractionation during mineralization of $\sim 3\%$ and almost exactly 3‰ more positive than observed for the non-nitrogen-fixing species¹².

Using an autoanalyser, nitrate and ammonium concentrations were then measured on water samples from a well. Total nitrogen content of ground waters were low, averaging only 0.71 mg l^{-1} . All nitrogen in the well-water was as nitrate; ammonium was

not detectable, confirming earlier observations of low nitrate contents and an absence of ammonium in both surface salt layers and groundwaters in a nearby salar¹³.

We measured the total plant-available nitrogen with depth in the soil profile under *Prosopis* canopies and the carbon to nitrogen ratios of live and dead fine roots in the upper rooting horizon. Available soil nitrogen was low, averaging $0.49\text{ }\mu\text{g g}^{-1}$ through the top 1.5 m of the soil profile. In the root-mat regions, available soil nitrogen was significantly higher ($0.88\text{ }\mu\text{g g}^{-1}$), but still sufficiently low to rank the soils as low in nitrogen. Carbon to nitrogen ratios of live and dead fine roots were 23.5 and 18.9 in the upper moist soil layers, respectively, indicating that some root nitrogen is being recycled in the soil. But the leaf and soil nitrogen isotope ratio data indicate that available soil nitrogen is not a significant component of the total *Prosopis* nitrogen budget.

Because total nitrogen contents of the groundwaters are low and the nitrogen isotope data indicated that essentially all of the nitrogen in *Prosopis* was derived from nitrogen-fixation activity, it would seem that there is effectively no nitrogen cycling in this ecosystem. This unusual nutrient pattern involves nitrogen production by nodules located in the moist soil layers 0.5–1.5 m below the surface, nitrogen translocation to the shoots where it is incorporated into leaves, essentially complete loss of this nitrogen during leaf abscission (with little if any recovery by the plant), and deposition of the nitrogen onto the salar surface where it remains undecomposed and not recycled. Given the high energetic costs associated with N_2 fixation as well as the low nitrogen contents of groundwater, it is surprising that so little of the *Prosopis* leaf nitrogen is retranslocated back into the plant before leaf abscission.

In terms of nitrogen, these plants behave as open systems, continually producing large amounts of nitrogen through nitrogen fixation activities and then annually depositing all of that nitrogen onto a surface litter layer that is never recycled through decomposition. Redistribution of this surface pool of litter may occur as highly irregular surface erosion events, but nevertheless it never enters the normal biological nitrogen cycle, making this Atacama Desert ecosystem unique in its nutrient cycling characteristics. □

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1. Fritz, P., Suzuki, O., Silva, C. & Salati, E. *J. Hydrol.* **43**, 161–184 (1981).
2. Rundel, P. A. *et al.* *Aliso* **13**, 1–49 (1991).
3. Mooney, H. A., Gulmon, S. L., Rundel, P. W. & Ehleringer, J. R. *Oecologia* **46**, 63–67 (1980).
4. Aravena, R. & Acevedo, E. in *The Current State of Knowledge on Prosopis tamarugo* (ed. Habit, M. A.) 251–256 (FAO, Santiago, 1985).
5. Margowitz, M., Aravena, R., Peña, H., Suzuki, O. & Grilli, A. *Ground Water* **28**, 513–517 (1990).
6. Alexander, G. V. & McAnulty, L. T. *J. Pl. Nutr.* **3**, 51–59 (1981).
7. Geyh, M. A. & Schleicher, H. *Absolute Age Determination: Physical and Chemical Dating Methods and Their Application* (Springer, Heidelberg, 1990).
8. Aguirre, J. J. & Wran, J. in *The Current State of Knowledge on Prosopis tamarugo* (ed. Habit, M. A.) 3–31 (FAO, Santiago, 1985).
9. Shearer, G. & Kohl, D. in *Stable Isotopes in Ecological Research* (eds Rundel, P. W., Ehleringer, J. R. & Nagy, K. A.) 342–374 (Springer, New York, 1988).
10. Prokisch, G. *Pl. Soil* **31**, 380–384 (1969).

11. Minagawa, M., Winter, D. A. & Kaplan, I. R. *Analyt. Chem.* **56**, 1859–1861 (1984).
12. Nadelhoffer, K. J. & Fry, B. *Soil Sci. Soc. Am. J.* **52**, 1633–1640 (1988).
13. Dingman, R. J. *U.S. Geological Survey Bulletin* 1219 (Washington DC, 1967).

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Predictable eye-head coordination during driving

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LARGE changes in the direction of gaze are made with a combination of fast saccadic eye movements and rather slower head movements. Since the first study on freely moving subjects¹, most authors have agreed that the head movement component of gaze is very variable, with a high 'volitional' component². But in some circumstances head and eye movements can be quite predictable, for example when a subject is asked to shift gaze as quickly as possible³. Under these conditions, laboratory studies have shown that the eye and head motor-systems both receive gaze-change commands, although they execute them in rather different ways^{3–6}. Here I reconsider the way gaze direction is changed during free movement, but in the performance of a task where the subject is too busy to exert conscious control over head or eye movements. Using a new portable and inexpensive method for recording head and eye movements, I examine the oculomotor behaviour of car drivers, particularly during the large gaze changes made at road junctions. The results show that the pattern of eye and head movements is highly predictable, given only the sequence of gaze targets.

If there are 'natural' patterns of oculomotor coordination that emerge in every day situations these should be detectable from the predictable way that head and eye movements co-vary. Let us assume that from time to time the brain specifies new gaze targets for the oculomotor system to fixate, and that their directions represent the commands to the system. An appropriate and severe test of the system's predictability would then be that the amplitudes and time courses of both the head and eye movements involved in every change of gaze should be dictated uniquely by the sizes of the gaze changes themselves. This study presents evidence in favour of this, and offers a simple model which generates realistic eye and head movements from gaze commands.

The device used to record eye movements consisted of video camera mounted on a cycling helmet. This took a 'head's eye'

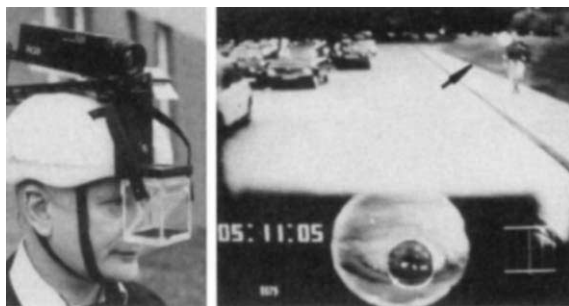


FIG. 1 Left, The scene-and-eye camera. A light-excluding shield has been removed for clarity. Right, VDU image showing a road scene with the inverted image of the eye at the bottom. The iris is ringed by a cursor which generates the gaze spot visible next to the pedestrian (indicated by arrow).

view of the world ahead. Attached to the camera was an arrangement of two mirrors and a lens which projected an enlarged image of the right eye onto the bottom of the camera's field of view (Fig. 1). The mirror in front of the eye was only 30% silvered, so that the driver's view was almost unimpeded. Recordings were made using available light, with a portable video cassette recorder and small battery pack. After the drive, the videotape had a clock signal added, and was viewed in combination with an image generated by an Acorn Archimedes computer. This contained a cursor program which modelled the outline of the iris on an eye rotating around its centre; the cursor could be moved with a tracker-ball so that it coincided exactly with the image of the iris on each video frame⁷. The angular coordinates used to generate the cursor were then taken as those of the direction of the axis of the eye itself, and they were in turn used to generate a spot on the image of the scene ahead, corresponding to the direction of view of the fovea (Fig. 1). The combined video and computer images were dumped frame-by-frame to a single-frame recorder (Panasonic AG-6720), and the coordinates of eye position stored simultaneously in the computer. Calibration consisted of fixating a series of points at known angular locations, and adjusting the cursor generator until the direction-of-view spot matched the appropriate point on the image to within 1°. Measurements were repeatable to 1°, and the absolute accuracy over a 40° field was about 2°. Head movements were measured directly from the video, by tracking distant objects across the calibrated monitor screen. The accuracy is similar to that of the eye movements. The temporal resolution is 50 Hz. Two subjects were each videotaped through three intersections like that shown in Fig. 2, providing a total of 112 saccadic gaze changes.

An example of the fixation points made while negotiating a road junction, with the corresponding horizontal eye and head

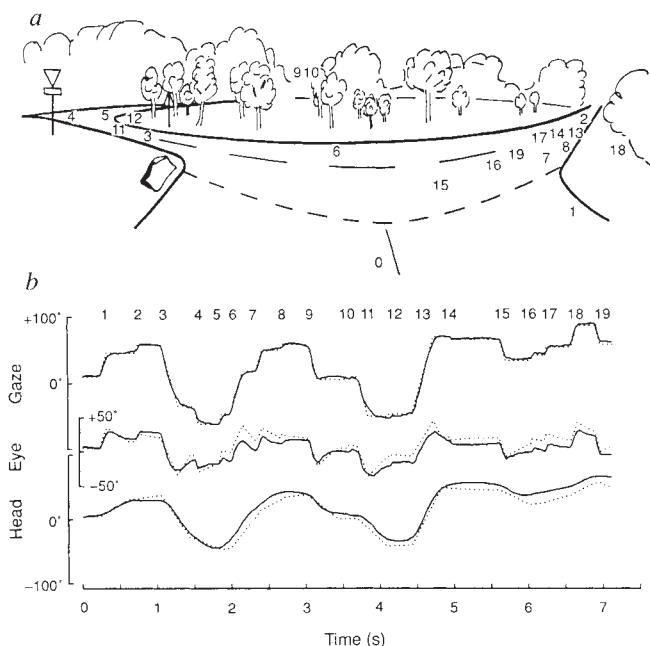


FIG. 2 a, Sequence of gaze fixation directions of subject M.L. during an approach to, and right turn at, an intersection. The sketch represents a 180° view and was made from photographs taken at a position corresponding to second 2 in b. The fixation points are taken from the video record. b, Horizontal eye, head and gaze movements during the sequence shown in a. Numbers above designate individual saccades. Right is represented vertically in all records. Head and gaze movements are relative to external coordinates, not the car, which was fully stationary only between saccades 10 and 14. The solid curves are the records themselves, and the dotted curves the outcome of the model described in the text and Fig. 3.

movements, is given in Fig. 2a,b. The records show the eyes looking left twice and right three times in a series of 19 saccades, made over 7 s. The gaze record, generated by adding the eye and head records, shows the familiar pattern of rapid changes of direction interspersed with periods of nearly stationary gaze (the slow movements between saccades 3 and 4, and 4 and 5 are due to the continued motion of the car relative to the fixation targets). Blinks accompanied seven of the larger saccades, a frequently reported finding. The overall form of the record clearly demonstrates three processes. Slow head movements produce the bulk of the gaze changes, with the eye saccades sharpening their beginnings. Thirdly, the vestibulo-ocular reflex (VOR) ensures that when the eye saccade is completed, the eye counter-rotates at a rate equal and opposite to the head movement⁸. This counter-rotation keeps gaze still between saccades.

To test whether the eye and head movements resulted from a single set of gaze-change commands, a computer model was set up. The input was the set of gaze-change amplitudes seen on the recording itself, with their times of occurrence (Fig. 3a). These gaze commands were interpreted by the eye- and head-movement systems in accordance with the following rules, which are broadly in line with current thinking on the way that eye and head movements are generated separately. (1) The same gaze change command is transmitted to both eye and head 'motors' simultaneously⁴. The command specifies the new gaze direction, in retinotopic coordinates, relative to the current direction of the visual axis. (2) The eye attempts to obey this command with an 'ideal' saccade that corresponds to the full extent of the command. However, the actual eye saccade is generally curtailed by the VOR resulting from the head move-

ment. For gaze changes greater than a few degrees the magnitude of the ideal eye saccade is principally determined by its duration (δt ; Fig. 3a) rather than its velocity, which has reached a maximum limiting value^{6,9,10}. My data, given in Fig. 3b, confirm the close relationship between saccade magnitude and duration. In the simulation shown in Fig. 2b, the velocity was taken to be a constant 400°s^{-1} , and the duration 2.5 ms per degree of gaze command. (3) The head begins to move at the same time as the eye. Unlike the eye movements, it is the velocity of the head that is determined by the size of the command (dh/dt , Fig. 3) with the duration of the movement fixed at about 0.4 s, independent of size³. Perhaps the most surprising result of this study was the close correlation between head velocity and gaze saccade size (Fig. 3c). In the simulation the velocity was set to 1.9°s^{-1} per degree of gaze command. Where one head movement begins before the previous one has finished, the effects are additive. (4) The VOR operates continuously, both during and between saccades. There is general agreement that this is not exactly what happens during saccades themselves¹¹⁻¹³ and that the head position signal is not subtracted directly from the eye position, but rather contributes to an internal feedback signal representing gaze direction. As the gaze target is attained during the saccade, the contribution required of the eye decreases, curtailing the eye movement. This mechanism requires the VOR itself to be switched off during a saccade, and reinstated at its completion. Although the latter is a more accurate physiological description of what occurs, the effect on the saccades themselves is very similar to continuous VOR, as originally proposed⁸; in particular, both models decrease the size of the contribution of the eye to the gaze change, and they slow its time course. The continuous VOR model is used here, because it is much simpler, involves fewer assumptions, and fits the actual data adequately.

This model is skeletal in that no attempt has been made to mimic fully the dynamics of either eye or head, and this results in a simulation that is rather more spiky than the actual record (Fig. 2b). Nevertheless, it is clear that the simulation fits the data sufficiently well for the 'single command' hypothesis to be regarded as basically correct. In particular, the head movement profile fits the actual movements with unexpected accuracy, and the amplitudes and velocity profiles of the eye saccades are also well approximated. The accuracy of the gaze amplitudes is of course not accidental, as these are the inputs to the model. But the model also predicts a constant velocity time course for the gaze changes (being mathematically identical to the ideal eye saccades), and this is very nearly attained, except at the ends of large saccades where the eye movements are slower than expected.

The overall appearance of the simulation can be improved cosmetically by making the head and eye accelerate more realistically, and by other minor changes. But these changes do not substantially improve the fit. The main discrepancy between the record and the model originates from the premature beginning of the head movement between saccades 4 and 5. This indicates a timing difference between head and eye commands, rather than a detail of execution. This early head movement shows up in Fig. 3c (arrow) as an exceptional over-fast head movement attributed to saccade 5 (Fig. 2); in records of the other five intersections there were a few occasions when the head led the first eye movement of a series by up to 150 ms, but strict synchrony (20 ms or less) was far more common. The other records had similar features to Fig. 2, and were generally well matched by the same model, although the second driver (J.B.) had eye movements that were faster than the first by about 20%. The only other two occasions on which the model seriously failed to match the data was when the eye reached its mechanical (or neural) limit at about 40° from the primary position. Here the head alone carried the gaze changes, so that these were slower (Fig. 3b, arrow), and their end-points less distinct. Thus the predictive power of the model is good, but not perfect.

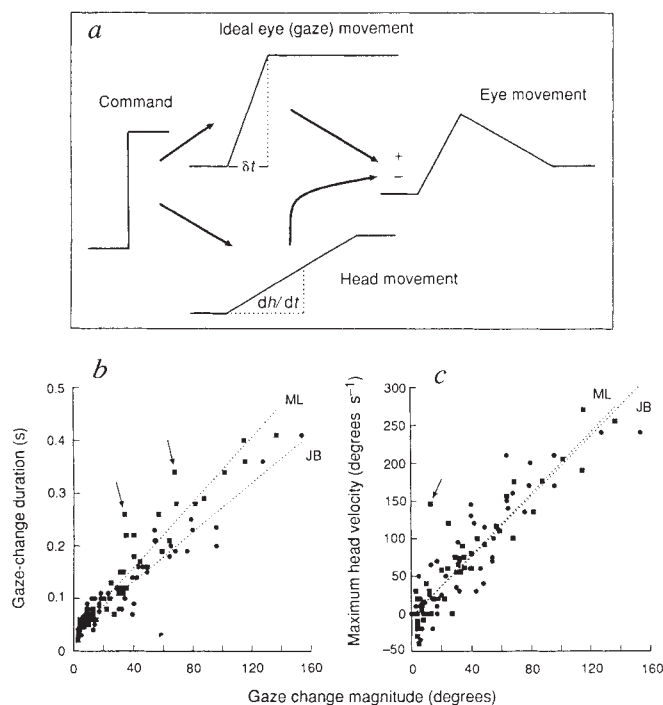


FIG. 3 a, Method of calculating head and eye movements from single gaze-change commands. Details in text. b, Relation between the durations of gaze saccades and their magnitudes, for 112 saccades at six intersections. ■, Driver M.L. (male, 49) $y=0.0031x+0.036$, $r=0.96$. ●, Driver J.B. (female, 30) $y=0.0023x+0.040$, $r=0.95$. Arrows indicate saccades in which the eye had reached its limit of travel. c, Relation between the angular velocity of head movements, measured 0.2 s after the beginning of each saccade, and magnitudes of the gaze movements, for the same saccades as in b. ■, $y=2.02x-3.3$, $r=0.92$. ●, $y=1.99x-3.7$, $r=0.91$. Arrow indicates the saccade in which the head began to move 0.2 s early (5 in Fig. 2b) giving a falsely high velocity.

The principal conclusion is that under circumstances where eye and head movements are generated unthinkingly, the two motor systems receive the same command at almost the same time. This seems to be the 'default' condition of the mechanism that directs our gaze. We can of course override it consciously by either making or suppressing head movements. Most of the time, however, the rules indicated here probably apply. □

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1. Collewijn, H. in *Control of Gaze by Brain Stem Neurons* (eds Baker, R. & Berthoz, A.) 13–22 (Elsevier, New York, 1977).
2. Zangemeister, W. H. & Stark, L. *Expl Neurol.* **77**, 563–577 (1982).
3. Zangemeister, W. H., Jones, A. & Stark, L. *Expl Neurol.* **71**, 76–91 (1981).
4. Bizzi, E., Kalil, R. E. & Tagliasco, V. *Science* **173**, 452–454 (1971).
5. Delreux, V., Abeele, S. V., Lefevre, P. & Roucoux, A. in *Brain and Space* (ed. Paillard, J.) 38–48 (Oxford Univ. Press, Oxford, 1991).
6. Becker, W. in *Vision and Visual Dysfunction*. Vol. 8. (ed. Carpenter, R. H. S.) 95–137 (Macmillan, Basingstoke, 1991).
7. Land, M. F., Marshall, J. N., Brownless, D. & Cronin, T. W. *J. comp. Physiol.* **A167**, 155–166 (1990).
8. Morasso, P., Bizzi, E. & Dichgans, J. *Expl Brain Res.* **16**, 492–500 (1973).
9. Yarbus, A. L. *Eye Movements and Vision* (Plenum, New York, 1967).
10. Carpenter, R. H. S. *Movements of the Eyes* (Pion, London, 1988).
11. Laurutis, V. P. & Robinson, D. A. *J. Physiol., Lond.* **373**, 209–233 (1986).
12. Tomlinson, R. D. *J. Neurophysiol.* **64**, 1873–1891 (1990).
13. Guitton, D. *Trends Neurosci.* **15**, 174–179 (1992).

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Deficiency of the 50K dystrophin-associated glycoprotein in severe childhood autosomal recessive muscular dystrophy

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X-LINKED recessive Duchenne muscular dystrophy (DMD) is caused by the absence of dystrophin, a membrane cytoskeletal protein^{1,2}. Dystrophin is associated with a large oligomeric complex of sarcolemmal glycoproteins^{3–10}. The dystrophin-glycoprotein complex has been proposed to span the sarcolemma to provide a link between the subsarcolemmal cytoskeleton and the extracellular matrix component, laminin^{7,9}. In DMD, the absence of dystrophin leads to a large reduction in all of the dystrophin-associated proteins^{4,9,10}. We have investigated the possibility that a deficiency of a dystrophin-associated protein could be the cause of severe childhood autosomal recessive muscular dystrophy (SCARMMD) with a DMD-like phenotype^{11–14}. Here we report the specific deficiency of the 50K dystrophin-associated glycoprotein (*M*, 50,000) in sarcolemma of SCARMMD patients. Therefore, the loss of this glycoprotein is a common denominator of the pathological process leading to muscle cell necrosis in two forms of muscular dystrophy, DMD and SCARMMD.

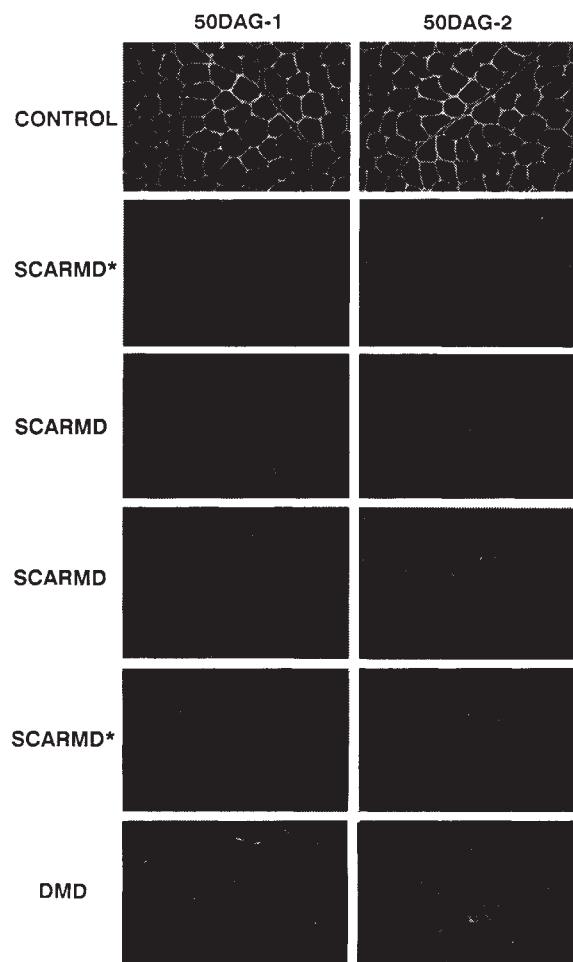


FIG. 2 Immunohistochemical analysis of 50DAG in biopsied skeletal muscle using a monoclonal antibody against 50DAG (50DAG-1) and a sheep polyclonal antibody affinity-purified against a 50DAG peptide (50DAG-2). Shown are (from top to bottom): 16-year-old male with no pathological changes in the skeletal muscle (CONTROL); 5-year-old male with SCARMMD; 10-year-old female with SCARMMD; 11-year-old male with SCARMMD; 11-year-old female with SCARMMD; and 8-year-old male with DMD (magnification, $\times 44$). * Siblings. METHODS. Serial transverse cryosections (7 μ m) were immunostained with IVD3₁, a monoclonal antibody against 50DAG, and a sheep polyclonal antibody affinity-purified against 50DAG peptide as described previously^{4–10}.

Figure 1 shows the immunohistochemical analysis of the muscle biopsy specimens using a monoclonal antibody against dystrophin and affinity-purified sheep polyclonal antibodies against 156K dystrophin-associated glycoprotein (156DAG), 59K dystrophin-associated protein (59DAP), 50K dystrophin-associated glycoprotein (50DAG), 43K dystrophin-associated glycoprotein (43DAG) and 35K dystrophin-associated glycoprotein (35DAG)^{4–10}. In normal skeletal muscle, antibodies against dystrophin and dystrophin-associated proteins (DAPs) stained the sarcolemma. In addition, we have found no abnormality of these proteins in the following neuromuscular diseases: limb-girdle muscular dystrophy, myotonic dystrophy, oculopharyngeal muscular dystrophy, facioscapulohumeral muscular dystrophy, non-Fukuyama type congenital muscular dystrophy, congenital fibre type disproportion, spinal muscular atrophy and amyotrophic lateral sclerosis (not shown). In DMD patients, dystrophin was absent and the immunostaining for all of the DAPs was greatly reduced in the sarcolemma (Fig. 1), except at the neuromuscular junction and in the sarcolemma of intrafusal muscle fibres (data not shown). In contrast, in four

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FIG. 1 Immunohistochemical analysis of dystrophin (DYS) and dystrophin-associated proteins in biopsied skeletal muscle. Shown are (from left to right): 16-year-old male with no pathological changes in the skeletal muscle (CONTROL); 5-year-old male with SCARM; 10-year-old female with SCARM; 11-year-old male with SCARM; 11-year-old female with SCARM; and 8-year-old male with DMD (magnification, $\times 28$). *, Siblings.

METHODS. Serial transverse cryosections ($7\ \mu\text{m}$) were immunostained with VIA4₂, a monoclonal antibody against dystrophin, and affinity-purified sheep polyclonal antibodies against 156DAG, 50DAP, 59DAG, 43DAG and 35DAG as described previously⁴⁻¹⁰. The diagnosis of SCARM was made on the basis of the following: (1) DMD-like phenotype affecting both males and females; (2) mode of inheritance compatible with an autosomal recessive disease; (3) North African patients; (4) elevated serum creatine kinase level; and (5) normal expression of dystrophin in the biopsied skeletal muscle analysed both by immunohistochemistry and by immunoblotting (Fig. 3)¹⁶.

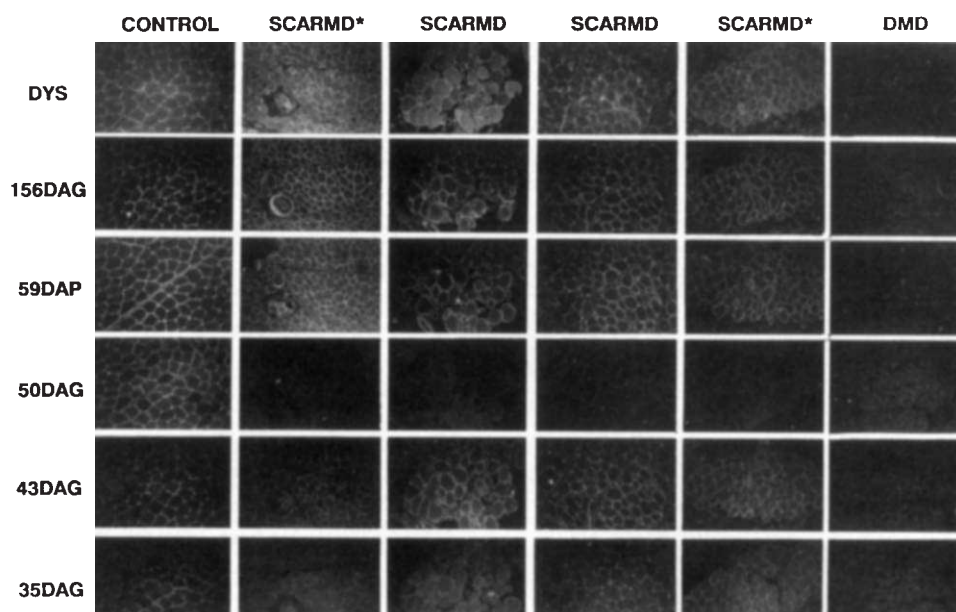
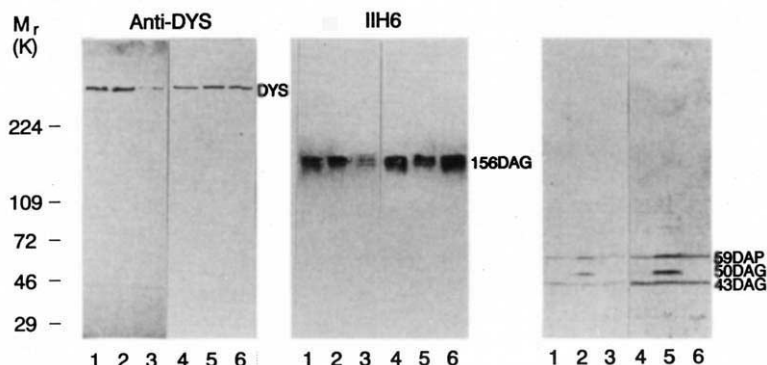


FIG. 3 Immunoblot analysis of the dystrophin (DYS)-glycoprotein complex in the SDS-extracts of the biopsied skeletal muscle. Shown are immunoblots stained with a polyclonal antibody against the last 10 amino acids of dystrophin (Anti-DYS), a monoclonal antibody against 156DAG (IIH6) and a cocktail of affinity-purified sheep polyclonal antibodies against 59DAP, 50DAG and 43DAG (Anti-DAPs), respectively. The affinity-purified sheep polyclonal antibody against 35DAG was not strong enough to stain the 35DAG in crude muscle extracts. Lanes 1 to 6 are samples from: 5-year-old male with SCARM; 41-year-old female with facioscapulohumeral muscular dystrophy; 10-year-old female with SCARM; 11-year-old male with SCARM; 16-year-old male with no pathological changes in the skeletal muscle; and 11-year-old female with SCARM (sister of patient in lane 1), respectively. Molecular weight standards ($\times 10^{-3}$) are shown on the left. **METHODS.** Cryosections ($20\ \mu\text{m}$) from skeletal muscle biopsy specimens were homogenized in 50 vols of SDS-extraction buffer (80 mM Tris-HCl, pH 6.8, 10% SDS, 0.115 M sucrose, 1% β -mercaptoethanol, 1 mM PMSF, 1 mM benzamidine and 1 mM EDTA) and incubated at 50°C for 10 min. After centrifugation, $10\text{-}\mu\text{l}$ samples were separated on 3-12% SDS-PAGE. The gel was stained with Coomassie blue and the density of the myosin



heavy chain band was measured using a computing laser densitometer (model 300S; Molecular Dynamics, Sunnyvale, CA). On the basis of this result, samples were run on 3-12% SDS-PAGE so that the amount of myosin heavy chain was equal for all specimens. Transfer to nitrocellulose membrane and immunostaining with antibodies were done as described³⁻¹⁰.

patients with severe childhood autosomal recessive muscular dystrophy (SCARM), including two siblings, immunostaining for the 50DAG was drastically diminished in the sarcolemma of all muscle fibres, including the neuromuscular junction and the sarcolemma of intrafusal muscle fibres, whereas immunostaining for dystrophin, 156DAG, 59DAP and 43DAG was preserved. Loss of 50DAG in SCARM patients was more severe than in DMD patients. Immunostaining for 35DAG in SCARM was reduced compared with normal control but was not as severely reduced as in DMD patients. Loss of 50DAG in the sarcolemma of SCARM patients was confirmed using three other specific antibodies against the 50DAG, a monoclonal antibody (Fig. 2), a sheep polyclonal antibody affinity-purified against a 50DAG peptide (Fig. 2) and an affinity-purified guinea-pig polyclonal antibody (data not shown)⁴⁻¹⁰.

To confirm the deficiency of the 50DAG in SCARM, skeletal muscle biopsy extracts were analysed by immunoblotting. Although dystrophin, 156DAG, 59DAP and 43DAG were detected, 50DAG was undetectable in all four SCARM patients

(Fig. 3). The affinity-purified antibody against the 35DAG was not strong enough to stain the 35DAG in crude muscle extracts. The deficiency of the 50DAG in SCARM muscle extracts was confirmed using two other antibodies against the 50DAG (data not shown).

SCARM (MIM number 253700)¹¹ is a progressive muscular dystrophy prevalent in North Africa¹¹⁻¹⁴. This disease shares several clinical features with DMD: mode of onset, rapid progression, hypertrophy of calves and extremely high serum creatine kinase levels in the initial stages of the disease. Dystrophin and dystrophin-related protein, an autosomal homologue of dystrophin¹⁵, are expressed normally in skeletal muscle in this disease^{13,16}. The structure and function of the dystrophin-glycoprotein complex (DGC) as a transsarcolemmal linker between the subsarcolemmal cytoskeleton¹⁷ and the extracellular component, laminin^{7,9}, suggest that a deficiency of a DAP could be the cause of an autosomal recessive muscular dystrophy with a DMD-like phenotype. Here we have demonstrated the specific deficiency of the 50DAG in the

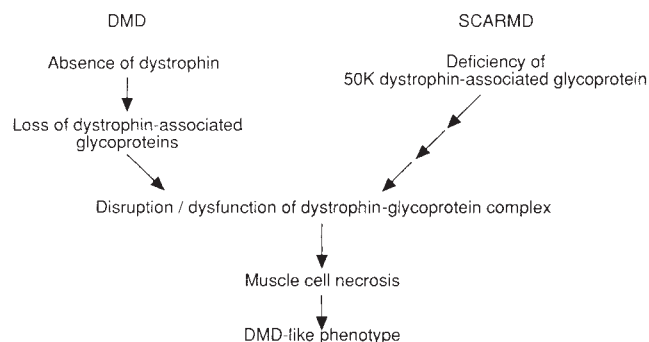


FIG. 4 Hypothetical scheme on the mechanism of muscle cell necrosis in severe childhood muscular dystrophies.

SCARMD sarcolemma. In the DMD sarcolemma, in contrast, the absence of dystrophin leads to a large reduction in all of the DAPs, including the 50DAG (Fig. 1, DMD panel)^{4,9,10}. Our results indicate that the loss of the 50DAG in the sarcolemma is a common denominator of the pathological process that leads eventually to muscle cell necrosis in two forms of muscular dystrophy, DMD and SCARMD.

On the basis of the function of the DGC mentioned above, we propose a hypothesis on the molecular mechanism of muscle cell necrosis in these two diseases where the disruption/dysfunction of the DGC plays a key role in the cascade of events leading to muscle cell necrosis (Fig. 4). In DMD, the absence of dystrophin leads to the loss of all DAPs, causing the disruption of the link between the subsarcolemmal cytoskeleton and the extracellular matrix that leads to sarcolemmal instability^{4,8-10}. In the case of SCARMD, in contrast, the deficiency of the 50DAG may severely disturb the function of the DGC and/or destabilize the DGC, also leading to sarcolemmal instability, which eventually causes muscle cell necrosis (Fig. 4).

The moderate reduction of the 35DAG in SCARMD could be secondary to the loss of the 50DAG. The 50DAG and 35DAG may form a tight subcomplex in the DGC and the loss of the 50DAG may cause a secondary reduction of the 35DAG in SCARMD. Dystrophin and 156DAG appeared reduced in the immunoblot analysis of the muscle specimen from the SCARMD patient with the most severe phenotype (lane 3 in Fig. 3). This suggests that the other components of the DGC could also be affected in the advanced stages of the disease.

Our results demonstrate the specific deficiency of the 50DAG in the SCARMD sarcolemma, leading to the reasonable hypothesis that this deficiency is causative of the disease. At present, the primary defect causing this deficiency is not known. It could be caused by a primary defect in the structure or expression of the gene for the 50DAG or could be due to a secondary effect of an unknown primary defect. Molecular biological and linkage analysis will be needed for the elucidation of the primary cause of SCARMD. But our results demonstrate that the diagnosis of SCARMD is now already possible by the immunochemical analysis of the 50DAG in muscle biopsy specimens. This will greatly aid the differential diagnosis between DMD and SCARMD. □

13. Ben Jelloun-Dellagi, S. *et al. Neurology* **40**, 1903 (1990).
14. Ben Hamida, M., Miladi, N., Turki, I. & Zaiem, H. *J. neurol. Sci.* **107**, 60-64 (1992).
15. Love, D. R. *et al. Nature* **339**, 55-58 (1989).
16. Khurana, T. S. *et al. Neuromusc. Dis.* **1**, 185-194 (1991).
17. Hemmings, L., Kuhlman, P. A. & Critchley, D. R. *J. Cell Biol.* **116**, 1369-1380 (1992).

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Amyloid β -peptide is produced by cultured cells during normal metabolism

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ALZHEIMER'S disease is characterized by the extracellular deposition in the brain and its blood vessels of insoluble aggregates of the amyloid β -peptide (A β), a fragment, of about 40 amino acids in length, of the integral membrane protein β -amyloid precursor protein (β -APP)¹. The mechanism of extracellular accumulation of A β in brain is unknown and no simple *in vitro* or *in vivo* model systems that produce extracellular A β have been described. We report here the unexpected identification of the 4K (M_r 4,000) A β and a truncated form of A β (~3K) in media from cultures of primary cells and untransfected and β -APP-transfected cell lines grown under normal conditions. These peptides were immunoprecipitated readily from culture medium by A β -specific antibodies and their identities confirmed by sequencing. The concept that pathological processes are responsible for the production of A β must now be reassessed in light of the observation that A β is produced in soluble form *in vitro* and *in vivo*² during normal cellular metabolism. Further, these findings provide the basis for using simple cell culture systems to identify drugs that block the formation or release of A β , the primary protein constituent of the senile plaques of Alzheimer's disease.

Human kidney 293 cells stably transfected with complementary DNA encoding the 695-amino-acid isoform of β -APP³ were metabolically labelled with ³⁵S-methionine. Immunoprecipitation of the conditioned medium with R1280, a high-titre antiserum against the synthetic peptide corresponding to residues 1-40 of A β (A β ₁₋₄₀; ref. 4), followed by electrophoresis and fluorography revealed two low-molecular-weight proteins migrating at ~4K and ~3K (Fig. 1a). The 4K band comigrated with radioiodinated synthetic A β ₁₋₄₀. The 4K and 3K bands were absent in precipitations with preimmune serum or R1280 preadsorbed with synthetic A β ₁₋₄₀ (Fig. 1a, b). Two additional high-titre polyclonal A β antibodies also precipitated the 4K peptide before but not after adsorption with A β ₁₋₄₀ (Fig. 1a). Medium from β -APP₆₉₅-transfected 293 cells contained substantially more of both the 4K and 3K peptides and the 90-100K soluble β -APP derivative (APP_s; refs 5-8) than did medium from untransfected cells (Fig. 1b, c). In contrast, none of these

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1. Hoffman, E. P., Brown, R. H. & Kunkel, L. M. *Cell* **51**, 919-928 (1987).
2. Koenig, M., Monaco, A. P. & Kunkel, L. M. *Cell* **53**, 219-228 (1988).
3. Campbell, K. P. & Kahl, S. D. *Nature* **338**, 259-262 (1989).
4. Ervasti, J. M., Ohlendorf, K., Kahl, S. D., Gaver, M. G. & Campbell, K. P. *Nature* **345**, 315-319 (1990).
5. Ohlendorf, K., Ervasti, J. M., Snook, J. B. & Campbell, K. P. *J. Cell Biol.* **112**, 135-148 (1991).
6. Ervasti, J. M., Kahl, S. D. & Campbell, K. P. *J. Biol. Chem.* **266**, 9161-9165 (1991).
7. Ervasti, J. M. & Campbell, K. P. *Cell* **66**, 1121-1131 (1991).
8. Ohlendorf, K. & Campbell, K. P. *J. Cell Biol.* **115**, 1685-1694 (1991).
9. Ibraghimov-Beskrovnaia, O. *et al. Nature* **355**, 696-702 (1992).
10. Ohlendorf, K. *et al. Neurology* (in the press).
11. McKusick, V. A. *Mendelian Inheritance in Man* 9th edn (The Johns Hopkins Univ. Press, Baltimore and London, 1991).
12. Ben Hamida, M., Fardeau, M. & Attia, N. *Muscle Nerve* **6**, 469-480 (1983).

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molecules were immunoprecipitated from medium of a human megakaryocytoid cell line (Dami⁹) that expresses no detectable β -APP (See Fig. 3a, lane 5). These results strongly suggest that the 4K and 3K peptides derive from β -APP.

To exclude the possibility that the 4K peptide represented a ~40-amino-acid fragment derived from the carboxy terminus of APP_s (residues ~573 to ~612 of β -APP₆₉₅; ref. 7), we precipitated medium of transfected 293 cells with an antibody raised against β -APP₅₇₆₋₅₉₅, yielding no 4K or 3K peptides but substantial amounts of APP_s (Fig. 2b, lane 2). An antibody (C7; ref. 10) against the β -APP C terminus likewise did not precipitate either peptide. Two antibodies directed at epitopes within the first 16 amino acids of A β precipitated the 4K but not the 3K peptide (Fig. 2b, lanes 4, 5). Two antibodies against A β epitopes C-terminal to residue 16 precipitated both 4K and 3K peptides (Fig. 2b, lanes 6, 7). These data, together with the molecular weights of the peptides, strongly suggest that the 4K peptide is A β and that the 3K peptide begins around residue 16 of A β near the secretory cleavage site of β -APP⁷ and ends near residue 40.

This conclusion was confirmed by separate labelled sequencing of the 4K and 3K molecules following ³H-phenylalanine labelling. The absolute positions and periodicity of the principal radioactive peaks were consistent with those predicted to arise from A β and from a truncated A β molecule (Fig. 2c). The main 4K species began at Asp 1 and therefore corresponds to A β . The major 3K species began at Val 18, the second residue of the 10K fragment produced during secretory cleavage of β -APP⁷. An additional species that began at Leu 17, the amino terminus of the 10K fragment, was present in about half the amount of the major Val 18 species. Minor fragments of the 4K and 3K molecules were detected beginning at Val -3 and Glu 11, respectively. These data demonstrate that 293 cells produce A β and a fragment comprising its C terminus.

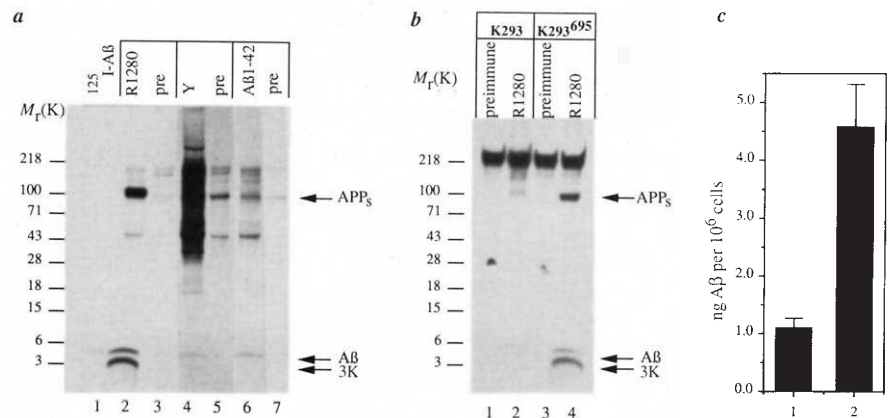
A β and the 3K peptide were produced by a variety of cell lines and primary cells, and their generation occurred irrespective of the particular β -APP isoforms being expressed. Kidney cells stably transfected with β -APP₇₅₁ generate amounts of 4K and 3K peptides similar to β -APP₆₉₅-transfected cells (Fig. 3a, lanes 2 and 3). A β was also detected in conditioned medium of human umbilical vein endothelial cells, human fetal mixed-brain cultures² and untransfected CHO cells (Fig. 3a and b).

In contrast to the other cell types, the mixed-brain cultures produced substantially more 4K than 3K species (Fig. 3b, lane 3). To investigate the solubility of the 4K and 3K peptides, medium of transfected 293 cells was centrifuged at 100,000g for 1 h. R1280 precipitation of the resulting supernatant and the SDS-solubilized pellet demonstrated complete recovery of the 4K and 3K peptides in the supernatant (Fig. 4a). Prolonged autoradiograph exposure of the R1280-precipitated lysates of 293 cells revealed no intracellular 4K and 3K peptides (Fig. 4b).

In contrast to current postulates, our findings demonstrate that A β is generated continuously as a soluble peptide during normal cellular metabolism. The failure to observe A β in biological fluids previously may relate both to a lack of sensitivity of earlier methods (like western blotting of plasma) and the assumption that the hydrophobic, membrane-spanning A β region would not normally occur extracellularly. The cells examined here were morphologically normal and unstained by vital dyes. We detected no intracellular A β in the same cells that showed readily detectable extracellular A β . Thus, generation of A β requires neither cellular injury nor aberrant proteolysis of β -APP, and the A β found in medium does not represent peptide simply released from dying cells. This is consistent with the observation that A β is present in the cerebrospinal fluid and plasma of normal humans². These findings raise the possibility that A β has a physiological function throughout life.

We detected no C-terminal fragments of β -APP in the medium under the conditions used here, whereas such fragments clearly have been detected intracellularly in tissues and cultured cells^{4,11-15}. One explanation for our results is that small amounts of A β -containing C-terminal fragments of β -APP (and perhaps even full-length β -APP) are slowly released during normal cellular metabolism, and that most of these are rapidly digested by extracellular proteases to yield A β and fragments thereof. The 3K peptide we detected in medium could represent an N-terminal fragment of the ~10K β -APP C-terminal peptide produced by secretory cleavage⁷ or may derive directly from proteolysis of A β in the medium. An alternative mechanism explaining our results could involve intracellular generation of A β from C-terminal β -APP fragments and its subsequent rapid release, making its intracellular detection difficult. In either mechanism, the C-terminal fragments that serve as the precursor

FIG. 1 Peptides of M_r 4K and 3K are specifically immunoprecipitated from culture medium by various antibodies to A β . **a**, Conditioned medium of ³⁵S-methionine-labelled kidney 293 cells stably transfected with β -APP₆₉₅ were precipitated with 3 different antibodies to A β . Lane 1, ¹²⁵I-labelled synthetic A β as a size marker; lane 2, R1280 (to A β ₁₋₄₀); lane 4, antiserum Y (to A β ₁₋₃₈; ref. 16); lane 6, anti-A β ₁₋₄₂ (ref. 18). A 4K peptide is precipitated by all three antibodies. In addition, a 3K peptide is precipitated by R1280 and very weakly by antiserum Y. All three antisera precipitate APP_s, the constitutively secreted, soluble β -APP derivative, one form of which is called protease nexin II (refs 5-8). Preadsorption (pre) of the antibodies (lanes 3, 5 and 7) abolished reactivity with the 4K and 3K peptides and markedly decreased reactivity with APP_s. The multiple higher-molecular-weight bands precipitated by antiserum Y are nonspecific in that they are not precipitated by other A β antibodies. **b**, Comparison of the amounts of 4K and 3K peptides produced by untransfected 293 cells (lane 2) and by 293 cells stably transfected with β -APP₆₉₅ (lane 4). R1280 precipitated substantially more 4K and 3K peptides from the transfected cells. Preimmune serum was negative (lanes 1 and 3). A 218K protein was nonspecifically precipitated in variable amounts by both preimmune and immune sera. **c**, Quantitation by an A β -specific ELISA² of the amounts of A β in the medium of untransfected (lane 1) and β -APP₆₉₅-transfected (lane 2) 293 cells. Results are

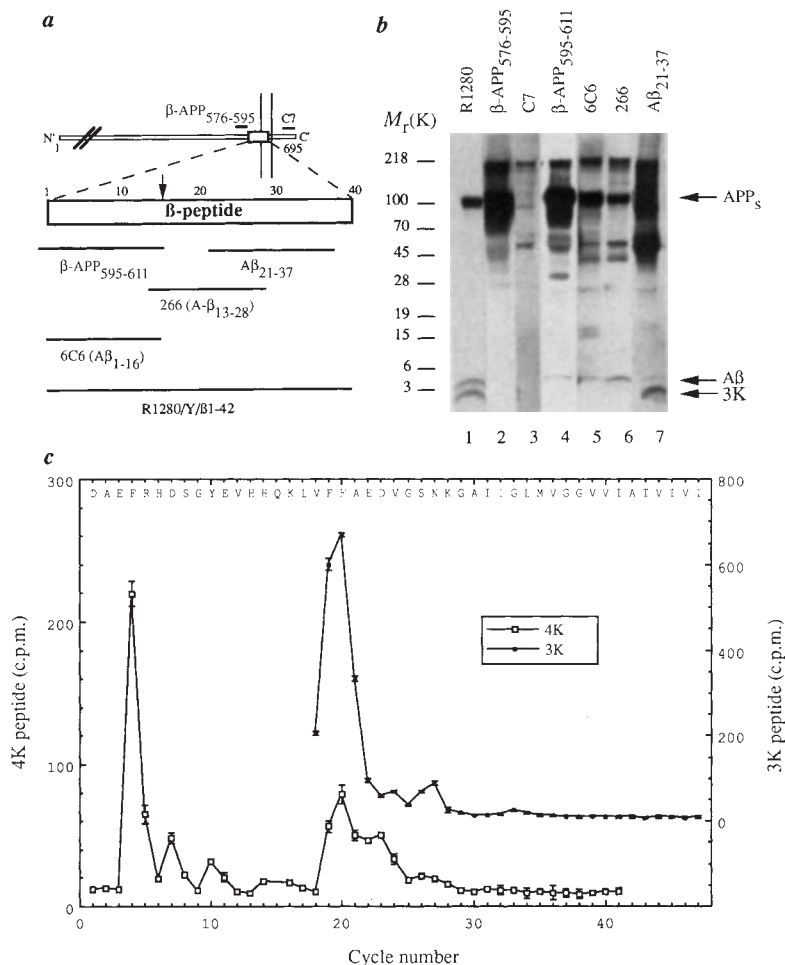


means $\pm$ s.d. of 5 determinations.

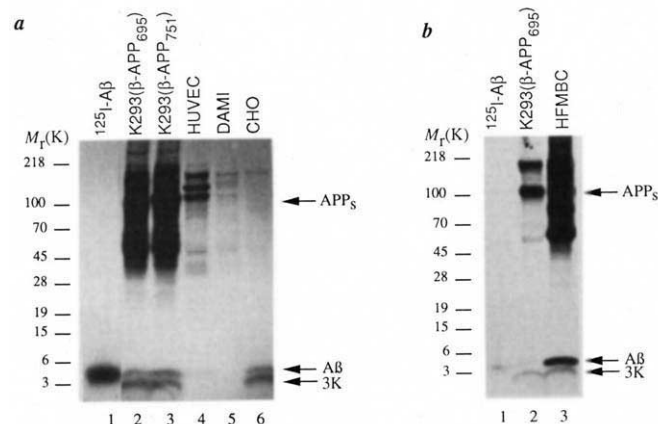
METHODS. Human kidney 293 cells were grown close to confluency ($\sim 1 \times 10^7$ cells) in 10-cm dishes and metabolically labelled for 16 h with 60 μ Ci ml⁻¹ of ³⁵S-methionine (NEN; 1,195 Ci mmol⁻¹) in methionine-free DMEM containing 10% (v/v) fetal bovine serum. Immunoprecipitations were done as described¹⁹. All antibodies were diluted 1:300. Immunoprecipitated proteins were separated on 10–20% Tris-Tricine gels¹⁴. For preadsorption, antibodies were incubated for 12 h at 4°C with the corresponding peptide antigen (15 μ g peptide per μ l antibody) and then added directly to medium.

FIG. 2 The 4K and 3K peptides are immunoprecipitated by various antibodies specific for different epitopes of A β . **a**, Schematic of β -APP and the A β region within it. Solid lines represent the regions against which antibodies were produced. Arrow indicates the secretory cleavage site⁷. Vertical lines on the β -APP schematic represent the transmembrane region. **b**, Conditioned media of radiolabelled kidney 293 cells stably transfected with β -APP₆₉₅ were immunoprecipitated with: lane 1, antiserum R1280 (to A β ₁₋₄₀) (ref. 4); lane 2, an antiserum to synthetic β -APP₅₇₆₋₅₉₅; lane 3, antiserum C7 (to β -APP₆₇₆₋₆₉₅) (ref. 10); lane 4, an antiserum to synthetic β -APP₅₉₅₋₆₁₁; lane 5, 6C6 (a monoclonal antibody recognizing A β ₁₋₁₆) (ref. 2); lane 6, 266 (a monoclonal antibody to A β ₁₃₋₂₈) (ref. 2); lane 7, an antiserum to A β ₂₁₋₃₇. To confirm the lack of immunoreactivity of antisera C7 and β -APP₅₇₆₋₅₉₅ with the 4K and 3K peptides, the gels were relatively overexposed. Higher molecular weight proteins other than APP_s precipitated by the various antibodies have not been characterized in detail and may represent nonspecific immunoreactivity. In particular, the ~218K protein is nonspecific. The APP_s precipitated by 266 (lane 6) probably represents a longer form of APP_s recently described in β -APP-transfected 293 cells²⁰. This form contains most or all of the A β ₁₃₋₂₈ region that was the immunogen for 266 (ref. 2). **c**, Radiolabelled sequencing of the 4K and 3K peptides produced major peaks of ³H-phenylalanine activity at positions consistent with A β molecules beginning at Asp 1 and at Val 18, respectively. Secondary signals at cycles 7, 22 and 23 in the 4K sequencing suggested the existence of an A β species beginning at Val -3. Similarly, secondary signals in the 3K sequencing at position 4 and at positions 9 and 10 were consistent with A β fragments beginning at Leu 17 and at Glu 11, respectively. The 3K peptide beginning at Val 18 may arise by exopeptidase removal of Leu 17 following the secretase-mediated cleavage of β -APP between Lys 16 and Leu 17 (ref. 7). Note that the data for the 3K molecule have been shifted 17 cycles to orient them properly with the A β sequence (that is, residue 1 corresponds to cycle 18). Data are expressed as mean counts per minute (c.p.m.) $\pm$ the s.d.; three counts of 5 min each were performed.

METHODS. Metabolic labelling and immunoprecipitations were performed as described in Fig. 1. For radioactive sequencing of the 4K and 3K peptides, a total of 10⁷ 293 cells stably transfected with β -APP₆₉₅ were metabolically labelled for 48 h with 1.8 mCi of L-[2,3,4,5,6-³H]phenylalanine (Amersham; 130 Ci mmol⁻¹, 360 μ Ci ml⁻¹). Media were collected and precipitated with R1280. As a marker to locate the 4K and 3K bands, medium from 2 $\times$ 10⁷ 293 cells stably transfected with β -APP₆₉₅ and metabolically labelled with ³⁵S-methionine were precipitated with R1280 and electrophoresed in parallel with the ³H-phenylalanine-labelled sample on a 10–20% Tris–Tricine gel.



The proteins were transferred to a Hyperbond polyvinylidene fluoride (PVDF) membrane (Pierce Instruments) essentially as described by Haass *et al.*¹⁹, except that the transfer buffer contained 20% (v/v) methanol. Autoradiography was for 4 days at -70 °C to visualize the ³⁵S-methionine-labelled proteins. The ³H-labelled 4K and 3K bands were cut out of the membrane separately by matching their positions with those of the ³⁵S-labelled 4K and 3K bands. PVDF strips were subjected to automated Edman chemistry in an Applied Biosystems Model 475A sequenator. Amino-acid anilinothiazolones were extracted with *n*-butyl chloride, transferred into 7-ml scintillation vials, and assayed for radioactivity after addition of 5 ml scintillation cocktail (Econofluor, NEN).



METHODS. Metabolic labelling and immunoprecipitations were carried out as described in Fig. 1. Conditioned medium from radiolabelled HFMBC was provided by T. Oltersdorf. Dami cells are a human megakaryocytoid cell line that was established from a 57-year-old man with megakaryoblastic leukaemia⁹.

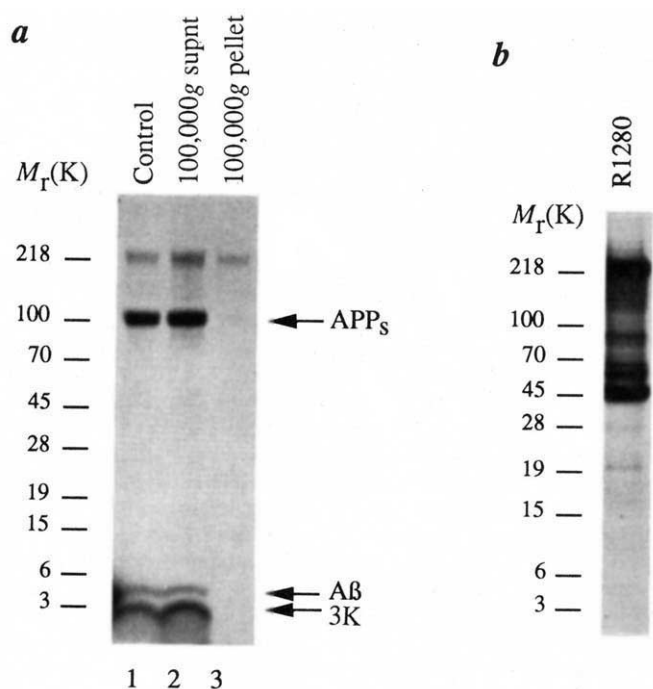


FIG. 4 Soluble A β is detected in cell culture supernatants but not in cell lysates. *a*, Conditioned medium of radiolabelled 293 cells transfected with β -APP₆₉₅ was precipitated with R1280 before (lane 1) or after (lane 2) a 100,000g spin. The pellet of the 100,000g spin was extracted with 1% (v/v) SDS and precipitated with R1280 (lane 3). *b*, Total cell lysates of $\sim 1 \times 10^7$ cells was precipitated with R1280, revealing no intracellular A β . METHODS. Conditioned medium (5 ml) from radiolabelled 293 cells transfected with β -APP₆₉₅ was centrifuged in a SW51 rotor at 100,000g at 4°C for 1 h. The supernatant was precipitated with R1280. The pellet was extracted in SDS-STEN buffer¹⁹, diluted in one volume of STEN buffer to lower the SDS concentration to 0.05%, and precipitated with R1280. R1280 routinely precipitated A β from media in the presence of 0.1% SDS. Cell extracts were prepared as described⁵.

of A β could arise from established β -APP processing pathways, namely by means of lysosomes^{13,14} or by an alternative cleavage during secretion, from a route not yet defined, or from a combination of pathways.

The finding that A β is generated *in vitro* as a soluble peptide that can readily be quantitated leads us to propose the use of various cultured cells, both neural and non-neural, as simple screens to identify synthetic and natural molecules which increase or decrease the production, release, or stability of A β .

Chronically enhanced production and/or decreased clearance of soluble, diffusible A β could lead to the gradual precipitation of aggregated non-diffusible A β in the form of spherical plaques and vascular deposits in Alzheimer's disease and Down's syndrome. Our findings indicate that several cellular sources could contribute to such deposits. The discovery of soluble A β in extracellular fluids fulfils a requirement of the hypothesis that A β in cerebral blood vessels and tissue could arise in part from a circulating source^{16,17}. □

10. Podlisny, M. B., Tolan, D. & Selkoe, D. J. *Am. J. Path.* **139**, 1423-1435 (1991).
11. Estus, S. et al. *Science* **255**, 726-728 (1992).
12. Nordstedt, C. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 8910-8914 (1991).
13. Golde, T., Esus, S., Younkin, L. H., Selkoe, D. J. & Younkin, S. G. *Science* **255**, 728-730 (1992).
14. Haass, C., Koo, E. H., Mellon, A., Hung, A. Y. & Selkoe, D. J. *Nature* **357**, 500-503 (1992).
15. Gabuzda, D. H., Busciglio, J. & Yankner, B. A. *Neurology* (abstr.) **42**, 304 (1992).
16. Joachim, C. L., Mori, H. & Selkoe, D. J. *Nature* **341**, 226-230 (1989).
17. Selkoe, D. J. *Neurobiol. Aging* **10**, 387-395 (1989).
18. Mori, H., Takio, K., Ogawara, M. & Selkoe, D. J. *J. Biol. Chem.* **267**, 17082-17086 (1992).
19. Haass, C., Hung, A. Y. & Selkoe, D. J. *J. Neurosci.* **11**, 3783-3793 (1991).
20. Anderson, J. P., Chen, Y., Kim, K. S. & Robakis, N. K. *J. Neurochem.* (in the press).

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Isolation and quantification of soluble Alzheimer's β -peptide from biological fluids

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CEREBRAL deposition of the β -amyloid peptide (A β) is an invariant feature of Alzheimer's disease. Since the original isolation and characterization of A β (ref. 1) and the subsequent cloning of its precursor protein²⁻⁵, no direct evidence for the actual production of discrete A β has been reported⁶⁻¹¹. Here we investigate whether A β is present in human biological fluids using antibodies specific for an epitope within A β that spans the site of normal constitutive cleavage^{12,13}. These antibodies were used to construct a sandwich-type enzyme-linked immunosorbent assay that detects A β in cerebrospinal fluid, plasma and conditioned medium of human mixed-brain cells grown *in vitro* (see also ref. 14). By affinity chromatography, we have purified and sequenced A β and a novel A β fragment from human cerebrospinal fluid and conditioned medium of human mixed-brain cell cultures. These findings demonstrate that A β is produced and released both *in vivo* and *in vitro*. These observations offer new opportunities for developing diagnostic tests for Alzheimer's disease and therapeutic strategies aimed at reducing the cerebral deposition of A β .

Monoclonal antibodies were raised against a fragment of A β comprising amino acids 13-28. Screening by radioimmunoassay showed that two of these antibodies, 266 and 067, immunoprecipitated radioiodinated A β ₁₋₂₈, but not A β ₁₋₁₆ or A β ₁₇₋₂₈, suggesting that reactivity of the antibodies depends on an intact sequence near the previously described site of constitutive secretory cleavage of β -amyloid precursor protein (β -APP) (refs 12, 13). These antibodies, together with a previously characterized antibody directed against A β ₁₋₁₆ (ref. 15) were used to develop a sensitive enzyme-linked immunosorbent assay (ELISA) specific for A β , with a sensitivity of ≤ 0.1 ng ml⁻¹. Various fragments of the β -APP, including the constitutively secreted form (APPs), were used to demonstrate the requirement

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1. Kang, J. et al. *Nature* **325**, 733-736 (1987).
2. Seubert, P. et al. *Nature* **359**, 325-327 (1992).
3. Selkoe, D. et al. *Proc. natn. Acad. Sci. U.S.A.* **85**, 7341-7345 (1988).
4. Tamaoka, A., Kalaria, R. N., Lieberburg, I. & Selkoe, D. J. *Proc. natn. Acad. Sci. U.S.A.* **89**, 1345-1349 (1992).
5. Weidemann, A. et al. *Cell* **57**, 115-126 (1989).
6. Oltersdorf, T. et al. *J. Biol. Chem.* **265**, 4492-4497 (1990).
7. Esch, F. et al. *Science* **248**, 1122-1124 (1990).
8. Sisodia, S. S., Koo, E. H., Beyreuther, K., Unterbeck, A. & Price, D. L. *Science* **248**, 492-495 (1990).
9. Greenberg, S. M., Rosenthal, D. S., Greeley, T. A., Tantravahi, R. & Handin, R. I. *Blood* **72**, 1968-1977 (1988).

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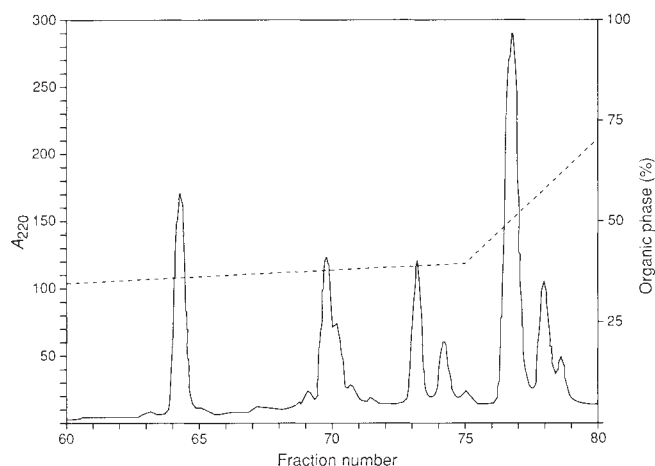
of a contiguous β -peptide sequence for reactivity in the assay (Table 1), thus allowing us to examine both *in vitro* and *in vivo* samples for the presence of $A\beta$ immunoreactivity.

Surprisingly, $A\beta$ immunoreactivity was identified in a variety of biological fluids from various species (Table 2). This reactivity appears to be specific on the basis of the findings presented later and in the accompanying report¹⁴, which demonstrates that conditioned medium from several cell types contains a peptide of M_r 4,000, which comigrates with authentic $A\beta$ and reacts only with $A\beta$ antibodies (including 266 and 10D5) and is increased in cells transfected with β -APP. ELISA quantitation of $A\beta$ in mixed-brain cell culture medium showed that levels

increase from initially undetectable to $\sim 4 \text{ ng ml}^{-1}$ after 72 h in culture. In addition, cerebrospinal fluid (CSF) or conditioned cell medium that is first preincubated with immobilized 266 or 067 antibodies is devoid of immunoreactivity in the ELISA, demonstrating that the signal generated is not due to nonspecific reaction of the biotinylated second antibody (data not shown).

To verify the identity of the $A\beta$ immunoreactivity measured in CSF and human mixed-brain cultures, immunoaffinity purifications from both sources were studied. In parallel experiments, either CSF or conditioned human mixed-brain culture medium was separately chromatographed on antibody 266 resin. ELISA data suggested that the $A\beta$ immunoreactivity in both CSF and the conditioned medium was completely captured by the resin. After elution, the specifically bound material was further purified by reversed-phase high-pressure liquid chromatography. A similar chromatographic profile was observed from both the CSF and human mixed-brain cell culture sources. The results of N-terminal sequence analysis and ELISA assay of the principal peaks detected by the absorbance at 220 nm of material derived from mixed-brain cultures are presented in Fig. 1. All peaks showing significant reactivity in the ELISA yielded N-terminal sequence data corresponding to the β -peptide (Fig. 1). Peaks 65, 70 and 79 also contained a previously undescribed β -peptide species beginning with Glu 11 which would probably not be detected by the 10D5 reporter antibody in our ELISA. Indeed, fraction 79, which contains only the $A\beta$ fragment starting with Glu 11, was silent in the assay (Fig. 1). The CSF-derived peak corresponding to human mixed-brain culture fraction 77 yielded $A\beta$ sequence beginning at Asp 1 and extending at least to residue 32 (Fig. 1).

As $A\beta$ was originally isolated and characterized from meningo-vascular and cerebral deposits on the basis of its extreme



Fraction number	N-terminal sequence							Initial yield*	$A\beta$ ELISA reactivity†
65	DAEFR	HDSGY	EVHHQ	KLVFF	AEDVG	SNKGA	I	~100	+
			EVHHQ	KLVFF	AEDVG	SNKGA	IIG	~70	
70	XAEFR	HDSGY	EVHHQ	KLVFF	AEDVG	SNKG(A)		~50	+
			XXXHQ	KLV				~2	
74‡	XQKTP	QIQVY	XRHP(P)	ENGKP	NILN			~15	-
75	DAEFR	HDSGY	EVHHQ	KLVFF	AE			~5	+
77§	DAEFR	HDSGY	EVHHQ	KLVFF	AEDV(S,G)	(S,G)NKGA	II	~120	+
79			EVHHQ	KLVFF	AEDXG	SN		~15	-

Sequences are given in one-letter amino-acid code; X, residue not identified.

* Calculated initial yield from microsequence analysis in pmol.

† A plus sign indicates more than 5 ng of material per fraction.

‡ Sequence matches mouse MHC class I β_2 -microglobulin N terminus, which is presumably present as a minor contaminant of antibody preparation 266.

§ Peak isolated from CSF gave identical sequence ending at position 32, and had an initial yield of 102 pmols.

FIG. 1 Isolation of β -amyloid peptide from CSF and human mixed-brain cell culture conditioned medium. CSF (3 l) or human mixed-brain cell conditioned media (4 l) was affinity-purified using an $A\beta$ monoclonal antibody affinity column followed by reversed-phase HPLC analysis. Shown is the absorbance profile at 220 nm (solid line) from human mixed brain cell culture material eluting at various concentrations of the organic phase (dashed line).

METHODS. Antibody 266 was coupled to Affi-Gel Hz Hydrazide resin (Bio-Rad) according to the manufacturer's instructions. For affinity chromatography 1 ml resin, estimated to contain $\sim 2 \text{ mg}$ of antibody, was packed in a $1 \times 10 \text{ cm}$ column and equilibrated with PBS. Neural tissue specimens from the cerebral cortex of 12–14-week-old fetal cadavers were rinsed twice in Hank's balanced saline solution (HBSS). Cortical tissue (2–3 g) was placed in 10 ml cold HBSS to which 1 mg DNase was added. The triturated suspension was filtered through Nitex nylon screens of $210 \mu\text{m}$ pore size and then $130 \mu\text{m}$. Cells were collected by centrifugation and resuspended in neuronal medium (MEM fortified with 10% fetal bovine serum, 1% glucose, 1 mM sodium pyruvate, 1 mM glutamine, 20 mM KCl). Polyethyleneimine-coated 100-mm dishes were seeded with 1.5×10^7 cells in 8 ml neuronal medium. The medium was collected and changed twice weekly. To 4 l of human mixed-brain cell conditioned medium, 4 mg leupeptin and 140 mg PMSF (in 4 ml isopropanol) were added. The material (CSF or conditioned medium) was passed through the 266 resin at a rate of $\sim 3 \text{ ml min}^{-1}$ at 4°C . The resin was washed extensively with PBS before eluting the specifically bound material with 8 ml of 0.2 M glycine, pH 2.0. The eluted material was subjected to two steps of reversed-phase liquid chromatography using a Vydac C4 ($0.21 \times 15 \text{ cm}$) column and a solvent system containing 0.1% trifluoroacetic acid (TFA) (buffer A) and 0.1% TFA/80% acetonitrile (buffer B). The affinity-purified sample was loaded onto the column at $200 \mu\text{l min}^{-1}$, then washed with 80% buffer A/20% buffer B for 42 min to equilibrate the column. A gradient from 20% to 70% buffer B was executed over 50 min at a flow rate of $50 \mu\text{l min}^{-1}$ while monitoring the absorbance of the eluant at 220 nm (A_{220}). Fractions of $100 \mu\text{l}$ were collected. Immunoblots probed with $A\beta$ antibody 10D5 gave a band corresponding to M_r 4,000 in fractions 11 and 12, followed by the light chain of the 266 antibody (data not shown). Fractions 11 and 12, positive for this 4K band, were pooled and rechromatographed under nearly identical conditions except that a 150-min gradient from 10 to 40% buffer 'B' was used, followed by a 20-min gradient from 40 to 100% buffer B. The peptides eluting in the regions of the chromatogram shown were microsequenced on an Applied Biosystems Model 477 protein sequencer with a microscale reaction cartridge and Applied Biosystem's MIFST program cycles.

TABLE 1 Specificity of A β Immunoassay

Peptide	Concentration (ng ml ⁻¹)	Cross reactivity (%) relative to 10 ng A β_{1-40} ml ⁻¹
A β_{1-40}	10	100
A β_{1-38}	10	93
A β_{1-28}	10	98
A β_{15-20}	1,000	—
A β_{13-20}	1,000	0.2
A β_{13-18}	1,000	—
β -APP(1-695)	100	0
β -APP(1-613)	100	—
β -APP (1-699) (KPI)	100	—

The preparation of monoclonal antibodies 10D5 or 6C6 which recognizes an epitope within the first 16 amino acids of the A β has been described¹³. Monoclonal antibodies 266 and 067 were generated against the synthetic peptide HHQKLFFAEDVGSNGGC (A β_{13-28}), which had been conjugated, via *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce), to anti-CD3 antibody (Boehringer Mannheim) before injection into A/J mice. The mice were immunized initially intraperitoneally (i.p.) with 360 μ g A β conjugate mixed with complete Freund's adjuvant. Fourteen days later the mice were boosted i.p. with the A β conjugate mixed with incomplete Freund's adjuvant. All subsequent boosts were i.p. with the A β conjugate mixed with phosphate-buffered saline (PBS) at 14-day intervals. After a total of six boosts, the mice were finally boosted intravenously with the A β conjugate mixed with PBS and fused 3 days later. The fusion of spleen cells with P3.653 myeloma cells was according to ref. 25, modified as described²⁶. Positive hybridoma supernatants were identified by their ability to immunoprecipitate ¹²⁵I-labelled A β_{1-28} and not ¹²⁵I-labelled A β_{1-16} or ¹²⁵I-labelled A β_{17-28} using a method employing sheep anti-mouse IgG coupled to CNBr-activated Sepharose 4B (ref. 27). Several positive clones were identified, subcloned and ascites prepared. All antibodies described were purified from ascites and judged as $\geq 90\%$ pure by Coomassie blue staining of SDS-polyacrylamide gels. A specific A β assay was developed using 1 μ g per well of 067 or 266 as a capture antibody and 10D5 as a biotinylated reporter antibody. The 10D5 was biotinylated using NHS-biotin (Pierce) according to a protocol supplied by the manufacturer. Immunoreactivity was visualized by the addition of streptavidin-alkaline phosphatase (Boehringer Mannheim) and addition of 4-methyl-umbelliferyl phosphate and quantitated in a Millipore Cytofluor 2350 fluorometer. Calibrators consisting of 0.1–10.0 ng ml⁻¹ of A β_{1-28} were used as standards. The various A β fragments were synthesized on an Applied Biosystems Peptide Synthesizer Model 430A. Purified β -APP forms were purified from transfected 293 cells as described¹². The A β region is composed of amino acids 672–713 of the 770-amino-acid-containing form of β -APP. This ELISA detects A β_{1-28} , A β_{1-38} and A β_{1-40} with comparable affinity and $\leq 0.2\%$ cross reactivity was observed with A β_{13-20} . Full-length or secreted APP were not cross reactive under these conditions. Data represent the mean of triplicate determinations. Standard deviations were less than $\pm 2\%$ in all cases.

insolubility, we were surprised to find apparently soluble A β in conditioned media and biological fluids. The perceived insolubility of A β together with the previous lack of specific and sensitive antibody reagents have, until now, prevented the identification of A β in biological fluids. The predominant form of immunoreactivity in at least one of the peaks was tentatively identified by electrospray ionization mass spectrometry as A β_{1-40} (peak 77; mass = $4,329.8 \pm 1.3$), which is in agreement with the primary structure of isolates from cortical and vascular deposits^{16,17}. These findings do not explain the additional fragments of A β that have been isolated from amyloid cores^{2,18}, nor do they answer the question of whether A β deposited in plaques and vessels is derived solely or partially from the soluble A β forms. The A β material isolated by immunoaffinity chromatography possesses either the expected sequence beginning with Asp at the N terminus, or the apparently novel fragment of A β beginning at Glu 11. At present, we do not have an explanation for the different high-pressure liquid chromatography retention times of A β species or for their comigration with the truncated species, but this could be due to

TABLE 2 Measurement of A β immunoreactivity

Source	A β (ng ml ⁻¹)		
	CSF	Plasma	Mixed brain cell culture
Human	2.5	0.9 $\pm$ 0.4	4.0
Dog	2.0	2.0	
Guinea-pig	2.5	4.5	
Rat	1.5	ND	

Human CSF was obtained from Universal Reagents, Indianapolis; dog and guinea-pig plasma and CSF were a gift from J. Clemens of Eli Lilly. Human plasma was collected in EDTA and was obtained from 11 healthy volunteers of ages ranging from 13–50 yr (31 ± 11 yr; mean $\pm$ s.d.). All samples were assayed immediately after collection or stored frozen at -20°C . The medium from mixed-brain cell culture was collected after 72-h incubation. The medium initially contains no detectable A β . All values were determined at least in duplicate, with standard deviations of $\leq 15\%$. The A β sequence of dog and guinea-pig is identical to human.²⁸ Rat A β (ref. 28) detection is about half as efficient as human on the basis of standard curves using synthetic rat A β_{1-28} (data not shown). ND, not determined.

different carboxy termini, conformations or states of aggregation.

It remains to be determined whether the truncated Glu 11 A β species has similar amyloidogenic properties. Physical chemical studies using synthetic variants of A β lacking the first 7–12 amino acids of A β suggest that these fragments might aggregate differently than longer species^{19,20}. These properties may be relevant to the apparent lack of detection of the shortened A β in senile plaques.

With the knowledge that A β is present in extracellular fluids, several important questions regarding the mechanism of β -amyloidosis in Alzheimer's disease can now be addressed. For example, the cellular pathways leading to A β production can now be dissected and the specific proteolytic enzymes identified. These studies can now be performed directly in tissue culture models and in laboratory animals. In addition, the effects on A β production of familial Alzheimer's disease point mutations can be tested^{21–24}. The ability to measure and study the metabolism of β -amyloid *in vitro* and *in vivo* should also facilitate the development of diagnostic tests and compounds directed at reducing the production of soluble A β . □

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- Glennier G. G. & Wong, C. W. *Biochem. biophys. Res. Commun.* **120**, 885–890 (1984).
- Kang, J. et al. *Nature* **325**, 733–736 (1987).
- Ponte, P. et al. *Nature* **331**, 525–527 (1988).
- Tanzi, R. E. et al. *Nature* **331**, 528–530 (1988).
- Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S. & Ito, H. *Nature* **331**, 530–532 (1988).
- Joachim, C. L., Mori, H. & Selkoe, D. J. *Nature* **341**, 226–230 (1989).
- Estus, S. et al. *Science* **255**, 726–728 (1992).
- Golde, T. et al. *Science* **255**, 728–730 (1992).
- Tamaoka, A. et al. *Proc. natn. Acad. Sci. U.S.A.* **89**, 1345–1347 (1992).
- Nordstedt, C. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 8910–8914 (1991).
- Buxbaum, J. D. et al. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6003–6006 (1990).
- Esch, F. S. et al. *Science* **248**, 1122–1124 (1990).
- Anderson, J. P. et al. *Neurosci. Lett.* **128**, 126–128 (1991).
- Haass, C. et al. *Nature* **359**, 322–325 (1992).
- Hyman, B. T. et al. *J. Neuropath. exp. Neurol.* **51**, 76–83 (1992).
- Joachim, C. L. et al. *Brain Res.* **474**, 100–111 (1988).
- Mori, H. et al. *J. Biol. Chem.* **267**, 17082–17086 (1992).
- Prelli, F. et al. *J. Neurochem.* **51**, 648–651 (1988).
- Zagorski, M. G. & Barrow, C. J. *Biochemistry* **31**, 5621–5631 (1992).
- Hilbich, C. et al. *J. molec. Biol.* **218**, 149–163 (1991).
- Goate, A. et al. *Nature* **349**, 704–706 (1991).
- Chartier-Harlin, M. C. et al. *Nature* **353**, 844–846 (1991).
- Levy, E. et al. *Science* **248**, 1124–1126 (1990).
- Murrell, J., Farlow, M., Ghetti, B. & Benson, M. D. *Science* **254**, 97–99 (1991).
- Köhler, G. & Milstein, C. *Nature* **256**, 495–497 (1975).
- Oi, V. T. et al. *Methods in Cellular Immunology* Ch. 17 (Freeman, San Francisco, 1990).
- Wang, R. W. et al. *J. Immun. Meth.* **18**, 157–164 (1984).
- Johnstone, E. M. et al. *Molec. Brain Res.* **10**, 299–305 (1991).

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Requirement for a functional *Rb-1* gene in murine development

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HUMAN retinoblastomas can occur both as hereditary and as sporadic cases. Knudson's proposal¹ that they result from two mutational events, of which one is present in the germ line in hereditary cases, has been confirmed by more recent molecular analysis, which has shown both events to involve loss or mutational inactivation of the same gene, *RB-1* (ref. 2). *RB-1* heterozygosity also predisposes to osteosarcoma, and *RB-1* allele losses are seen in sporadic lung, breast, prostate and bladder carcinomas³⁻⁷. *RB-1* is expressed in most, if not all, tissues and codes for a nuclear phosphoprotein which becomes hypophosphorylated in the G0 growth arrest state and in the G1 phase of the cell cycle². To gain a further insight into the role of *RB-1* we and other groups^{8,9} have generated mice carrying an inactivated allele of the homologous gene, *Rb-1* (ref. 10), by gene targeting¹¹. We report here that young heterozygous mice do not appear abnormal and do not develop retinoblastoma at a detectable frequency. However, homozygous mutant embryos fail to reach term and show a number of abnormalities in neural and haematopoietic development. Broadly similar results are reported by the other groups^{8,9}.

E14 Embryonic Stem (ES) cells, derived from strain 129/Ola (ref. 12) and cultured in BRL medium¹³ or LIF-supplemented

medium¹⁴⁻¹⁶ were used to generate mutated clones carrying an insertion into exon 19. Two targeting vectors were used to generate mutant clones. The isogenic targeting vector 129Rb-hyg was used to generate clones sRb66 and sRb96. As previously observed with this vector¹⁷ a high proportion of hygromycin-resistant clones (27 out of 36 tested) resulted from the correct integration of *hyg* into the 19th exon of *Rb-1* without the need for a further step to enrich for homologous recombination. Clone sRb123 was generated using a closely related nonisogenic vector containing 18 kilobases (kb) of homology with a *neo* cassette inserted into exon 19 and a herpes simplex virus thymidine kinase (*tk*) cassette added to the 3' end of the genomic sequence (Fig. 1). From 3,600 colonies resistant to G418 (determined by control plates), 240 were doubly resistant to G418 and ganciclovir, of which 227 were screened by Southern blotting analysis. In three of these clones, one copy of the *Rb* gene was found to carry the predicted modification. Because the insertions carry translation termination codons in all three reading frames and polyadenylation signals¹⁸⁻²⁰, the *Rb-1* reading frame is truncated upstream of the coding sequences for T antigen-binding domain number 2, which is essential for the function of the gene product²¹. Even in the event of deletion of exon 19 by aberrant splicing, a frameshift would be generated. We will therefore consider the targeted alleles, which we designate *Rb-1*^{119hyg} and *Rb-1*^{119neo}, as null alleles throughout the text, although residual activity of the truncated gene cannot be formally excluded.

Germ line chimaeras were obtained from clones sRb66, sRb96 and sRb123 after injection into F2(C57BL/6×CBA) or C57BL/6 blastocysts. Offspring were analysed by Southern blotting to confirm presence of the mutated allele, and mice heterozygous (+/−) for the mutation were mated to generate animals homozygous (−/−) at the mutant locus (Fig. 1b). Some of these animals were descended from matings of chimaeras to strain 129/Ola females, and were thus coisogenic with strain 129/Ola; others were generated from matings to BALB/c or FVB females, resulting in a variable, crossbred genetic background.

The effects of *Rb-1* genotype were similar in mice derived from all three targeted clones. In contrast to the situation in humans², none of 82 +/− mice ranging in age up to 7 months displayed any sign of retinoblastoma or, indeed, any other

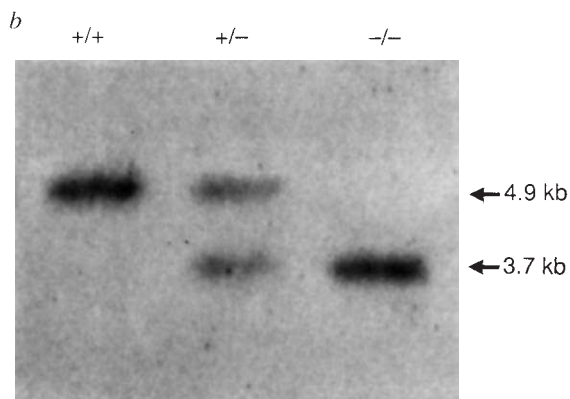
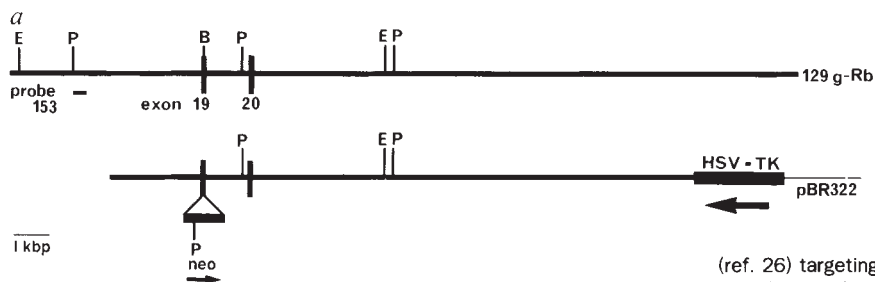


FIG. 1 A strategy for the disruption of the retinoblastoma gene. *a*, *Rb-1* locus around exons 19 and 20 (black boxes, numbered according to the human *RB-1* gene organization; presence of additional exons within the depicted region is not excluded). The PNS

(ref. 26) targeting vector used to generate clone sRb123 was created by inserting a *pgk-neo* cassette²⁷ into the *Bgl*II site of exon 19 and adding a *pgk-tk* cassette to the 3' end of the *Rb-1* homology (1.8 kb *Bgl*II-*Eco*RI *HSVtk* fragment²⁸ in the *Sma*I site of a *pgk* expression vector²⁹). The external 5' probe (a 0.45 kb fragment from a *Pst*I/*Pvu*II double digest) used for Southern analysis is indicated. Thick lines, genomic DNA. Thin lines, plasmid DNA. B, *Bgl*II; E, *Eco*RI; P, *Pst*I. *b*, Typical Southern analysis of progeny after restriction with *Pst*I, and probing with the external probe. The positions of bands corresponding to the wild-type *Rb-1* allele (4.9 kb) and the *neo*-containing allele (3.7 kb) are indicated.

METHODS. Electroporation was done as previously described¹⁹. Targeted cells were selected after 1 day in 200 μ g ml⁻¹ G418 and after 4 days counterselected in 2 μ M ganciclovir. Genomic DNA was prepared from ES cells, tail biopsy material or, as is the case in *b*, from yolk sac of the midgestation conceptus. It was then digested, separated in a 0.8% agarose gel by electrophoresis, transferred to a Hybond-N+ filter (Amersham) and hybridized according to manufacturers' conditions.

abnormality; nor were retinoblastomas macroscopically detected in 38 chimaeras ranging in age up to 11 months. However, there were no $-/-$ animals among 30 liveborn offspring of $+/- \times +/-$ matings, a statistically significant deficiency compared to the expected mendelian proportion ($P = (0.75)^{30} = 0.0002$). We conclude that $-/-$ mice die *in utero*.

To investigate the nature of this embryonic lethality, embryos ranging from day 10.5 to day 12.5 postcoitum (p.c.) were collected. A total of 11 litters was analysed. These contained 83 pups of which 41 were $+/-$, 17 $-/-$ and 25 $+/+$. There was no statistically significant deficiency of $-/-$ embryos compared with the expected mendelian proportion ($\chi^2 = 0.90$, $P > 0.05$). However, 12 resorptions were seen, of which four could be genotyped, two being $-/-$ and two $+/-$. We therefore cannot exclude the possibility that some $-/-$ embryos die before the stages analysed.

Several abnormalities were seen to various degrees in these homozygous embryos, but none was universally present. These included a light appearance to the conceptus in its intact yolk sac (in contrast to the heterozygote, where yolk sac blood vessels were clearly visible) and an altered morphology of the head. Features of this altered morphology included a more prominent fourth ventricle, compression of the forehead and a distinctly smaller frontal lobe (Fig. 2). These features may be due to altered local growth, developmental retardation or both, as the mutant embryos bear a resemblance to wild-type embryos slightly earlier in development. Of the 17 $-/-$ embryos, 11 appeared pale or retarded, two appeared externally normal, but sectioning revealed abnormalities, three appeared normal but were not sectioned and one appeared normal both visually and histologically. An increased rate of cell death by apoptosis²² was evident in the spinal cord, myelencephalon, pontine flexure, and particularly in the spinal ganglia of $-/-$ embryos (Fig. 3). In one animal degeneration extended into the prosencephalon. Following submission of our manuscript, our attention was drawn to the observations of Lee *et al.*⁸ that the increased cell death is predominantly in the intermediate zone and not in the ventricular zone, and that ectopic mitosis also occurs in this

region in the homozygote. On re-examination of our preparations we confirm these findings. The light appearance of the $-/-$ embryos suggested a disruption of normal haematopoiesis; we therefore investigated several aspects of this process (Fig. 4). Aberrant erythropoiesis in $-/-$ embryos was confirmed by the presence of abnormal erythrocytes in fetal blood (Fig. 4b), a deficiency in mature nucleated red cells in the yolk sac vessels (Fig. 4d) and abnormal proliferation of immature erythrocytes in the liver (Fig. 4f). This was accompanied by a failure of haematopoiesis in liver cultures (Fig. 4h) and, in some cases, an increase in liver megakaryocyte levels (Fig. 4f) or in myeloid cell numbers (not shown). No abnormalities were detected in the eyes of $-/-$ embryos. Effects of both differences in time of fertilization and variation in genetic background on the time of onset of abnormalities may contribute to the variable phenotype seen at these embryonic stages, and may explain small differences between the phenotype reported here and by the other two groups^{8,9}, such as the fact that Jacks *et al.* saw no abnormalities until 13.5 days⁹, and our detection of a deficiency in the earlier, yolk sac lineage. The only inbred $-/-$ embryo we have so far studied was highly abnormal and undergoing resorption at 10.5 days. Further work will clarify whether the time of onset of abnormality is less variable in inbred $-/-$ embryos.

The present work demonstrates that inactivation of one copy of *Rb-1* has little or no effect in the young mouse and in particular does not lead to the development of retinoblastoma at detectable frequency. Similar results are reported by the other groups^{8,9}, although brain tumours⁸ and, specifically, pituitary tumours⁹ have been found in animals older than our heterozygotes. Rather than reflecting a basic biological difference between murine *Rb-1* and human *RB-1*, this may be a consequence of differences between mouse and human such as a reduced number of target cells or faster developmental rate in the mouse. The failure of homozygous mice to develop to term indicates a role for *Rb-1* in normal development. Expression of *Rb-1* messenger RNA is widespread in the mouse embryo. Maximal expression occurs at 12.5–14.5 days' gestation, with the highest levels in liver, brain and spinal column¹⁰, and in the liver high levels of *Rb-1* protein are present in megakaryocytes and blood-forming islands²³. This correlates well with the tissue-specificity and time of onset of the abnormalities reported here. *Rb-1*-expressing cells have in common that they are in the course

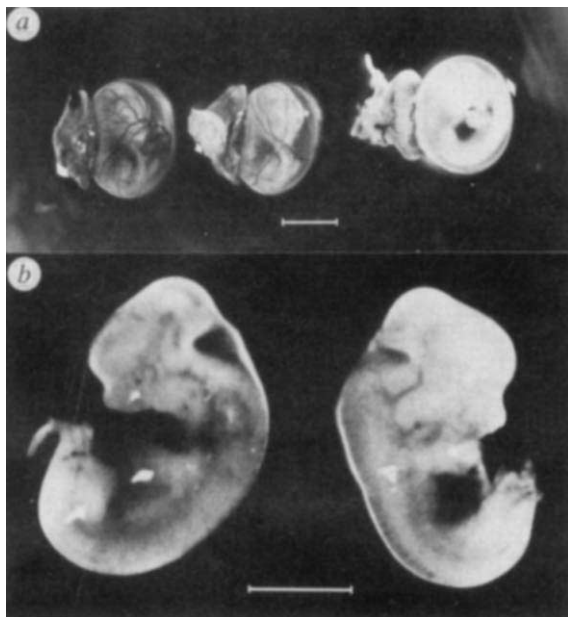


FIG. 2 External morphology of day 12.5 embryos. *a*, Left to right $+/+$, $+/-$ and $-/-$ litter mates before dissection from yolk sac. *b*, $-/-$ (left) and $+/-$ (right) litter mates after dissection from yolk sac. The $-/-$ embryo carries a more prominent fourth ventricle, compressed forehead and smaller frontal lobe. Scale bars, 0.5 cm.

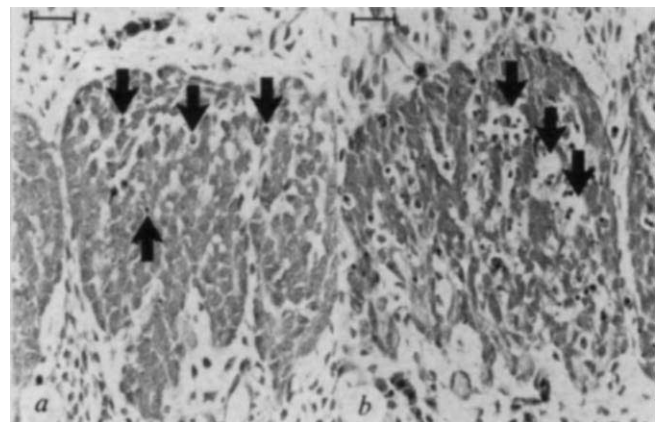


FIG. 3 Histology of spinal ganglia in 11.5-day p.c. $+/-$ and $-/-$ litter mate embryos. *a*, $+/-$: only occasional apoptotic cells, identified by their pyknotic and fragmented nuclei and dense eosinophilic cytoplasm²², are present (some arrowed). *b*, $-/-$: widespread apoptosis is seen in confluent zones (some arrowed). Haematoxylin and eosin stain. Scale bars, 50 μ m. METHODS. Conceptuses were dissected into embryo, yolk sac and placenta. Embryos were fixed in buffered formalin, embedded and sectioned at 5 μ m. Yolk sac was used to prepare DNA for genotyping as in Fig. 1.

of differentiation, suggesting a general role for *Rb-1* in the maturation of precursor cells²³. Furthermore, it has been hypothesized²² that one of the functions of *Rb-1* is to maintain cells in a growth-arrest state, wherein they show a reduced susceptibility to apoptosis and that when a functional *Rb-1* gene product is absent cells assume a state in which there is a high

turnover and an aberrant pattern of apoptosis is generated. These considerations may explain the expansion of inappropriate haematopoietic lineages and the increased levels of neural apoptosis and ectopic mitosis reported here and by the other groups^{8,9}. These do not involve the eye, as does somatic homozygosity for a nonfunctional *RB-1* allele in humans, but involve tissues in which abnormalities have not been reported in retinoblastoma patients. This may be a consequence of the difference between germline and somatic homozygosity. Homozygous mice may die before the retina reaches a susceptible stage. Rescue of homozygous or doubly targeted cells^{21,24,25} by wild-type cells in chimaeras may allow study of the effect of homozygosity on later stages of retinal development. □

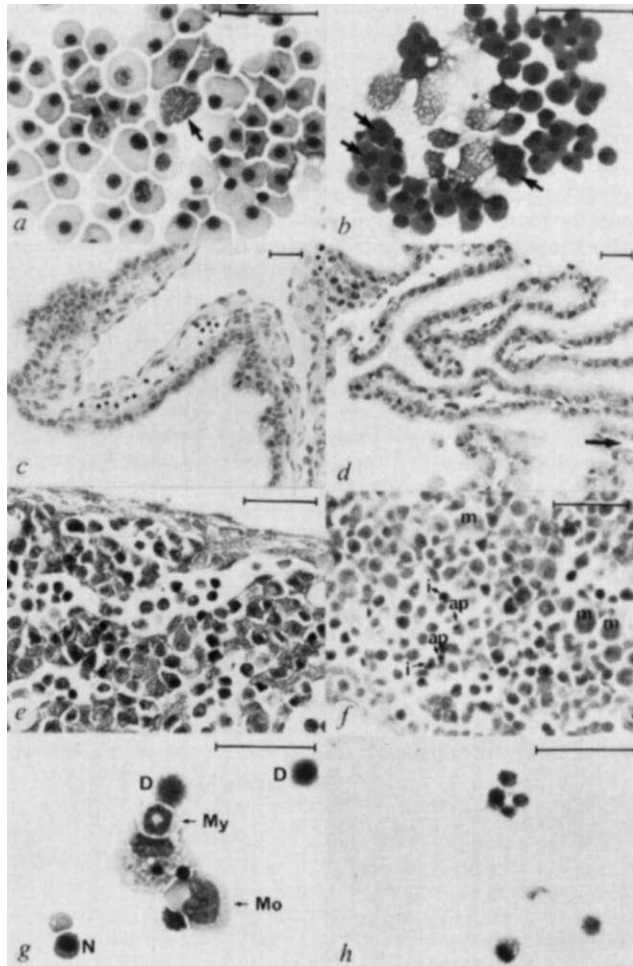


FIG. 4 Haematopoiesis in $+/+$ and $-/-$ litter mate embryos. *a, b*, Cytopsin preparations of fetal blood at 12.5-days p.c. *a*, $+/+$: predominantly nucleated red cells with round nuclei at various stages of condensation with occasional immature white cell (arrow). *b*, $-/-$: lower ratio of red to white cells, the former having more densely stained cytoplasm of reduced volume and pyknotic nuclei that are in some cases irregular (arrows). *c, d*, Yolk sac histology at 12.5-days p.c. *c*, $+/+$: capillaries contain numerous nucleated red cells. *d*, $-/-$: the yolk sac membrane is thinner with empty capillaries (one arrowed) and few red cells. *e, f*, Liver histology at 11.5-days p.c. *e*, $+/+$: regular trabecular structure with nucleated red cells in sinusoids. *f*, $-/-$: trabecular structure irregular with dilated sinusoids containing red cells, some of which are irregular (i) and some apoptotic (ap). Increased numbers of megakaryocytes (m) are present. *g, h*, 12.5-days p.c. liver culture supernatants. *g*, $+/+$: various white cell types are present including cells of the myeloid (My) and monocytoid (Mo) lineages, as well as occasional nucleated red cells (N) and debris (D). *h*, $-/-$: only debris present. *a, b, g, h*, Giemsa stain; *d, e, f, g, h*, haematoxylin and eosin stain. Scale bars, 50 μ m. METHODS. Fetal blood obtained from the umbilical vessels was used for cytopsin preparations. Histological preparations were made as described in Fig. 3; where yolk sac was used for histology the embryo was used for genotyping. Fetal liver cultures were established by passing the tissue through a 19G needle and plating in RPMI 1640 medium³⁰ supplemented with 10% fetal calf serum and 30% (v/v) of the same medium conditioned by the spleen cell line S27.1 (A.R.C. *et al.*, manuscript in preparation). After 4 days, cytopsin preparations were made from culture supernatants.

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- Knudson, A. G. *Proc. natn. Acad. Sci. U.S.A.* **68**, 820-823 (1971).
- Weinberg, R. A. *Trends biochem. Sci.* **15**, 199-202 (1990).
- Lee, E. Y. *et al. Science* **241**, 218-221 (1988).
- Yokota, J. *et al. Oncogene* **3**, 471-475 (1988).
- Stratton, M. R. *et al. Br. J. Cancer* **60**, 202-205 (1989).
- Shew, J. Y., Ling, N., Yang, X. M., Fodstad, O. & Lee, W.-H. *Oncogene Res.* **4**, 205-214 (1989).
- Cance, W. G., Brennan, M. F., Dudas, M. E., Huang, C.-M. & Cordon-Cardo, C. *New Engl. J. Med.* **323**, 1457-1462 (1990).
- Lee, E. Y.-H. *et al. Nature* **359**, 288-294 (1992).
- Jacks, T. *et al. Nature* **359**, 295-300 (1992).
- Bernards, R. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 6474-6478 (1989).
- Capocchi, M. R. *Science* **244**, 1288-1292 (1989).
- Hooper, M., Hardy, K., Handyside, A., Hunter, S. & Monk, M. *Nature* **326**, 292-295 (1987).
- Smith, A. G. & Hooper, M. L. *Dev. Biol.* **121**, 1-9 (1987).
- Moreau, J.-F. *et al. Nature* **336**, 690-692 (1988).
- Smith, A. G. *et al. Nature* **336**, 688-690 (1988).
- Williams, R. L. *et al. Nature* **336**, 684-687 (1988).
- Te Riele, H., Robanus Maandag, E. & Berns, A. *Proc. natn. Acad. Sci. U.S.A.* **89**, 5128-5132 (1992).
- Thomas, K. R. & Capocchi, M. R. *Cell* **51**, 503-512 (1987).
- Kaster, K. R., Burgett, S. G., Rao, R. N. & Ingolia, T. D. *Nucleic Acids Res.* **11**, 6895-6911 (1983).
- Adra, C. N., Boer, P. H. & McBurney, M. W. *Gene* **60**, 65-74 (1987).
- Hu, Q., Dyson, N. & Harlow, E. *EMBO J.* **9**, 1147-1155 (1990).
- Dive, C. & Wyllie, A. H. in *Frontiers in Pharmacology: Cancer Chemotherapy* (eds Hickman, J. A. & Tritton, T. T.) (Blackwell Scientific, Oxford, in the press).
- Szekely, L. *et al. Cell Growth Differ.* **3**, 149-156 (1992).
- Te Riele, H., Robanus Maandag, E., Clarke, A., Hooper, M. & Berns, A. *Nature* **348**, 649-651 (1990).
- Mortensen, R. M., Conner, D. A., Chao, S., Geisterfer-Lowrance, A. A. T. & Seidman, J. G. *Molec. cell. Biol.* **12**, 2391-2395 (1992).
- Mansour, S. L., Thomas, K. R. & Capocchi, M. R. *Nature* **336**, 348-352 (1988).
- McBurney, M. W. *et al. Nucleic Acids Res.* **19**, 5755-5761 (1991).
- Colbere-Garapin, F., Chousterman, S., Horodniceanu, F., Kourilsky, P. & Garapin, A.-X. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3755-3759 (1979).
- Van der Lugt, N., Robanus Maandag, E., Te Riele, H., Laird, P. W. & Berns, A. *Gene* **105**, 263-267 (1991).
- Moore, G. E., Gerner, R. E. & Franklin, H. A. *J. Am. med. Assoc.* **199**, 519-524 (1967).

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Inefficient positive selection of T cells directed by haematopoietic cells

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SUCCESSFUL intrathymic differentiation of $\alpha\beta$ TCR⁺ T cells depends on positive selection of CD4⁺CD8⁺ thymocytes by thymic major histocompatibility complex (MHC) molecules¹⁻⁹. Positive selection allows the maturation of only those T cells capable of restricted antigen recognition in the context of the hosts' MHC alleles. Studies of normal or T-cell receptor-transgenic mice engrafted with MHC-different bone marrow or thymuses support the conclusion that positive selection is directed by MHC molecules

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expressed on non-haematopoietic cells, presumably thymic epithelial cells^{1-3,8-10}. Here we present contrary evidence that class I MHC molecules expressed by haematopoietic cell types direct positive selection of CD8⁺ T cells, though at a reduced rate compared with positive selection directed by thymic epithelial cells. The identity of cell types that direct positive selection bears directly on mechanistic models of the process, including the idea that thymic epithelial cell MHC molecules uniquely present specialized peptides that mediate positive selection, and the notion that thymic epithelial cells express unique differentiation-inducing cell surface molecules.

Mice deficient for class I cell-surface expression owing to a mutation in the β_2 -microglobulin genes (β_2m^-) genes are severely deficient for the production of functional T-cell receptor (TCR) α/β CD8⁺ T cells^{11,12}. By using chimaeric animals to control the site of class I expression, we have now determined the relative contribution of class I expression by different tissues in directing positive selection. To determine whether haematopoietic cells need to express β_2m for differentiation of CD8⁺ T cells, we prepared chimaeras in which irradiated β_2m^+ C57BL/6 recipients were repopulated with fetal liver cells from MHC-matched β_2m^- or β_2m^+ mice¹³. Fetal liver cells were used in these and other chimaeras to rule out outgrowth of mature T cells that can contaminate bone marrow preparations. Normal

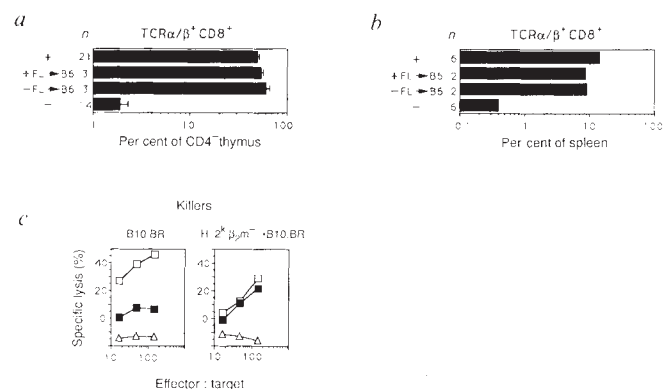
frequencies of donor-derived TCR α/β CD8⁺ thymocytes (Fig. 1a) and splenocytes (Fig. 1b) developed in both types of chimaera and were functional, as shown by their ability to produce an efficient allo-specific cytotoxic T-lymphocyte (CTL) response (Fig. 1c).

The previous results suggest that class I molecules on thymic epithelial cells can direct positive selection, but do not address whether haematopoietic cells can also direct positive selection. Therefore, we prepared chimaeras in which irradiated β_2m^- recipients were repopulated with fetal liver cells from MHC-matched β_2m^- or β_2m^+ mice. The frequency of TCR α/β CD8⁺ thymocytes or spleen cells in β_2m^- recipients of β_2m^+ fetal liver, although lower than in β_2m^+ mice, was about four times higher than in the control chimaeras in which both the recipient and fetal liver cells were β_2m^- ($P < 0.05$ by Student's *t*-test) (Fig. 2a-c). These cells expressed both CD8 α and CD8 β (data not shown). They were functional, as they mounted strong allo-specific CTL responses (Fig. 2d), mediated by CD8⁺ cells (data not shown). In contrast, cells from β_2m^- recipients of β_2m^- fetal liver did not mount detectable CTL responses (Fig. 2d).

Although the preceding results suggest that haematopoietic cells can inefficiently direct positive selection, it remained possible that transferred β_2m ¹⁴ from donor β_2m^+ cells could reconstitute functional expression of the class I heavy chains that

FIG. 1 Functional CD8⁺ T cells develop efficiently from β_2m^- precursors in a β_2m^+ thymus. *a, b*, Irradiated B6 recipients were repopulated with fetal (embryonic day 16) liver cells. Donor fetal liver (FL) cells were from intercrosses of (B6 $\times$ 129)F₄ (H-2^b) mice, and were β_2m^+ (+) or β_2m^- (-) as indicated. After reconstitution (9-15 weeks), the frequencies of CD8⁺CD4⁻TCR α/β ^{hi} cells were determined among thymocytes (*a*) or splenocytes (*b*). Nonchimaeric β_2m^+ and β_2m^- mice, from (B6 $\times$ 129)F₂₋₅ crosses or from ((B6 $\times$ 129) $\times$ B6) (third backcross generation), served as controls. For thymocytes, mature CD8⁺ cells were first enriched by depleting CD4⁺ cells (~90% of thymocytes), as described¹¹. *c*, Generation of β_2m^- CTL from ($\beta_2m^- \rightarrow \beta_2m^+$) chimaeras. This experiment used chimaeras of the H-2^k haplotype, which were generated by repopulating irradiated H-2^k β_2m^+ mice with H-2^k β_2m^- fetal liver cells. Chimaeric spleen cells (right panel) or control B10.BR spleen cells (left panel), were stimulated in primary MLC with B6 spleen cells. Before the cytotoxicity assay, samples of the CTL were depleted of class I positive cells (■), or not (□, △) to assess whether the effector cells were donor-derived (β_2m^-). The surviving cells, without adjusting for losses, were tested for lysis of B6 (■, □) or control B10.BR (△) target cells. In each panel, the number of individually analysed mice (*n*) is indicated.

METHODS. Fetal liver chimaeras were constructed as follows: on day -1 C57BL/6 and B10.BR recipients received 200 μ g anti-NK1.1 monoclonal antibody (PK136). This treatment is necessary to promote engraftment of β_2m^- haematopoietic cells which we previously showed are otherwise rejected by host natural killer cells²⁴. On day 0, the animals were irradiated (980 rads, ¹³⁷Cs source) and inoculated intravenously with 7-10 million fetal liver cells (embryonic day 16). The frequency of CD8⁺CD4⁻ $\alpha\beta$ TCR^{hi} cells was determined by three-colour immunofluorescence essentially as described¹¹. Fluorescein-conjugated anti-K^b antibody (AF6-88.5; Pharmingen) was included in the first staining step so that during analysis donor-



derived cells could be electronically gated on the basis of their class I (K^b) staining profile. In all cases, contaminating host-derived cells accounted for <13% of the total. In *a*, the data shown are the means and standard errors of the mean. In *b*, two chimaeras of each type were analysed; individual determinations were within 50% of the mean. In *a*, at least 10,000 cells were counted for each sample; in *b*, at least 50,000 cells were counted. CTL were generated in mixed lymphocyte cultures as described²⁵. Portions of each culture were depleted of class I-positive cells by treating with anti-K^b antibody (15-1-5P) and complement for 45 min at 37 °C. Live cells were recovered by passage over a Ficoll gradient and assayed for CTL activity against concanavalin A blast target cells²⁵ without adjusting for cell losses.

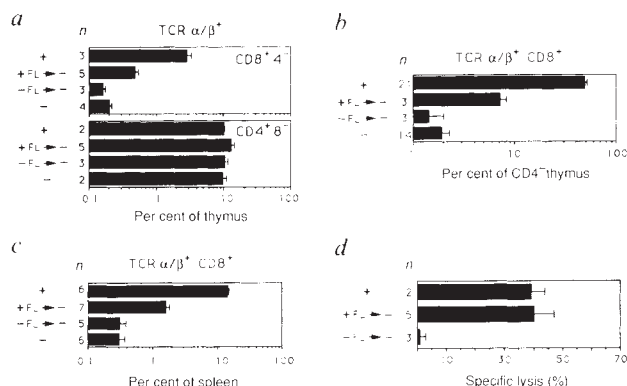


FIG. 2 CD8⁺ T cells develop inefficiently from β_2m^+ precursors in a β_2m^- thymus. *a-c*, Day-16 fetal liver cells were used to repopulate irradiated β_2m^- (B6 $\times$ 129)F₃ or F₄ mice. The donor fetal liver cells were from intercrosses of H-2^b (B6 $\times$ 129)F₃ or F₄ mice, and were β_2m^+ or β_2m^- as indicated. After reconstitution (9-27 weeks), the frequencies of CD8⁺CD4⁻ $\alpha\beta$ TCR^{hi} cells were determined among thymocytes (*a, b*) or spleen cells (*c*). In *b*, CD8⁺CD4⁻ thymocytes were enriched before analysis by depleting CD4⁺ cells. In *a*, the frequency of CD4⁺CD8⁻ $\alpha\beta$ TCR^{hi} thymocytes was also determined as a control. Data from at least 30,000 cells were collected in *a* and *c*; at least 10,000 cells were collected from most animals in *b, d*. CD8⁺ T cells from (H-2^b $\beta_2m^+ \rightarrow$ H-2^b β_2m^-) chimaeras are functional. Spleen cells from the indicated fetal liver chimaeras, or control B6 mice were stimulated in MLC with BALB/c (H-2^d) spleen cells, and tested for lysis of BALB/c concanavalin A blast target cells. No lysis of control B6 target cells was observed (not shown). Results from multiple animals at a single effector to target ratio (30/1) are shown. Complete titration curves failed to reveal significant activity of cells from ($\beta_2m^- \rightarrow \beta_2m^-$) chimaeras.

may be expressed at low levels on recipient β_2m^- thymic epithelial cells^{11,15,27}. This could be tested with chimaeras where β_2m^+ haematopoietic cells and β_2m^- host cells express different class I alleles; the restriction specificity of the resulting CTL can be used to infer the identity of the cells that directed positive selection^{1,2}.

Groups of lethally irradiated parental MHC-type β_2m^+ or β_2m^- recipients were repopulated with (C57BL/6 $\times$ B10.BR) F_1 (H-2^{k/b}) fetal liver cells. The reconstituted animals were primed *in vivo* and boosted *in vitro* against BALB/c minor histocompatibility (minor H) antigens presented on (BALB.K $\times$ BALB.B) F_1 (H-2^{k/b}) splenocytes. CTL from ($F_1 \rightarrow$ H-2^b β_2m^+) control chimaeras showed a very strong preference for H-2^b restriction, as expected (Fig. 3). In contrast, CTL from ($F_1 \rightarrow$ H-2^b β_2m^-) chimaeras showed essentially no bias in MHC restriction, that is they lysed BALB.B and BALB.K target cells equally well (Fig. 3). This pattern of lysis was similar to that exhibited by control ($F_1 \rightarrow$ H-2^{b/k} β_2m^+) chimaeras. These results argue strongly that

positive selection in ($\beta_2m^+ \rightarrow \beta_2m^-$) chimaeras is not directed by reconstituted thymic epithelial class I molecules. The fact that positive selection in β_2m^- recipients is dependent on β_2m expression by haematopoietic cells (Fig. 2) strongly indicates that haematopoietic cell class I molecules can direct positive selection.

Because the target cells in these experiments (concanavalin A blasts) do not express class II MHC molecules, the lytic activity must be class I MHC-restricted. The failure of the CTL to lyse control (B6 $\times$ B10.BR) F_1 targets shows their specificity for minor H antigens. A control experiment (not shown) showed that CTL from ($F_1 \rightarrow$ H-2^{b/k} β_2m^+) chimaeras that lyse BALB.B and BALB.K targets are nonoverlapping sets, arguing strongly against the possibility that a nonclassical class I molecule shared by BALB.B and BALB.K restricts part of this minor H-specific CTL response. Therefore, it is highly unlikely that in ($F_1 \rightarrow$ H-2^b β_2m^-) chimaeras, a nonclassical class I molecule shared by the H-2^b and H-2^k haplotypes directs positive selection of minor H specific CTL. This possibility is also unlikely as it would require that the nonclassical class I molecule, but not the classical ones, reach the cell surface and can be functionally assembled with donor-derived β_2m in chimaeras.

It is important to emphasize that although our results indicate that haematopoietic cell class I molecules are able to direct positive selection, selection by these cells was considerably less efficient than selection by β_2m^+ thymic epithelial cells. In this respect, our results agree with earlier conclusions that thymic epithelial cells are highly proficient at directing positive selection^{1-4,6-8,10,16} and contrast with a study that arrived at the opposite conclusion¹⁷. Thus, our results are not incompatible with most studies using conventional chimaeric mice, because a limited degree of positive selection directed by haematopoietic cells might be missed in such animals.

This issue has also been examined more quantitatively with mice transgenic for a D^b-restricted T-cell receptor. In those studies, transgenic H-2^b bone marrow cells differentiating in H-2^k hosts yielded only about 5% of the number of mature CD8⁺ T cells as in H-2^b hosts⁴. It is difficult to ascertain from the experiment whether this small number of mature CD8⁺ T cells could represent a low level of positive selection directed by haematopoietic cells, or instead corresponds to background levels of detection. If the latter is true, three explanations can be considered to reconcile their data with ours. Perhaps positive selection by haematopoietic cells requires a particular range of TCR-MHC affinity, and the affinity of the transgenic TCR happens to fall outside of that range. Alternatively, the substantial number of T cells expressing endogenously encoded receptors in TCR-transgenic mice may successfully compete with haematopoietically selected T cells for a limiting number of thymic niches¹⁸. Finally, the transgenic TCR may be selected by an MHC-bound peptide that happens not to be expressed by haematopoietic cells (though our results suggest that selecting peptides are not generally restricted in expression to thymic epithelial cells; see later).

It is not clear which haematopoietic cell type(s) directs positive selection in our chimaeras. Among haematopoietic cells in the thymic cortex, where positive selection is believed to occur, only cortical thymocytes and macrophages express detectable class I molecules¹⁹. Class I⁺ cortical thymocytes are an abundant cell type in the cortex, but class I expression by these cells is relatively weak. This could account for the inefficient positive selection we observed. Because class II molecules are not expressed by thymocytes, it will be interesting to determine whether CD4⁺ T-cell development occurs in (class II⁺ $\rightarrow$ class II⁻) haematopoietic chimaeras^{20,21}.

Our results argue strongly against models in which positive selection depends on specialized peptides presented uniquely by thymic epithelial cell class I molecules⁷. That model suggested that the TCR-self MHC affinity is augmented by peptides unique to thymic epithelial cells, resulting in sufficient affinity to trigger

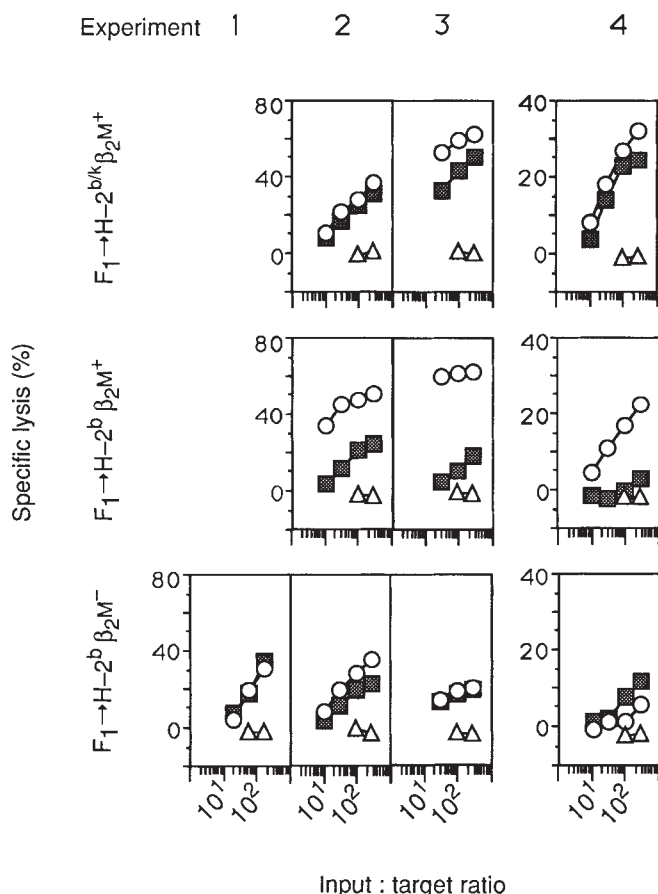


FIG. 3 MHC restriction specificity of CD8⁺ T cells that develop in β_2m^- recipients of β_2m^+ fetal liver cells. Donor fetal liver cells were from crosses of B6 and B10.BR mice, that is H-2^{b/k}. Irradiated recipients were H-2^{b/k}/ β_2m^+ (B6 $\times$ B10.BR) F_1 , H-2^b β_2m^+ (B6) or H-2^b β_2m^- (from (B6 $\times$ 129) F_4 crosses). After reconstitution (17–25 weeks), the chimaeras were primed *in vivo*, and subsequently boosted *in vitro*, with (BALB.B $\times$ BALB.K) F_1 spleen cells, which present BALB/c minor H antigens in the context of H-2^b and H-2^k. Minor H-specific CTL were tested on BALB.B (○) and BALB.K (■) concanavalin A blast target cells to measure H-2^b-restricted and H-2^k-restricted CTL, respectively. (B6 $\times$ B10.BR) F_1 concanavalin A blast target cells (△), syngeneic to the responding T cells, served as a negative control. Cells from a fixed fraction of each culture were titrated in the assay; hence the data is displayed as the ratio of the number of spleen cells initially cultured (input) to the number of target cells. Chimaeras of the type ($F_1 \rightarrow$ H-2^k β_2m^+) showed only modest bias for H-2^k restriction, as had been previously reported²⁶; therefore ($F_1 \rightarrow$ H-2^k) chimaeras were not very informative and are not presented here.

positive selection⁷. Our results therefore support an alternative hypothesis, that positive selection of immature thymocytes may require a lower triggering threshold than does negative selection or activation of mature T cells.

The possibility that thymic epithelial cells might normally provide an essential differentiation-inducing signal to T cells, independently of the TCR, is not ruled out by our data. It is possible that when the thymocytes' TCR engages class I on a haematopoietic cell, the delivery of the differentiation-inducing signal by a neighbouring thymic epithelial cell can still proceed inefficiently. A precedent is the finding that mature T-cell clones can recognize antigen on one cell, and receive a costimulatory signal from another cell²². That this type of mechanism might operate in positive selection agrees with recent findings that Thy1⁺ cells from nude mice can achieve functional maturation in an allogeneic thymus, yet exhibit the non-thymic, donor type MHC restriction specificity²³. □

4. Kisielow, P., Teh, H. S., Bluthman, H. & von Boehmer, H. *Nature* **335**, 730-733 (1988).
5. Sha, W. C. et al. *Nature* **336**, 73-76 (1988).
6. Marusic-Galesic, S., Stephany, D., Longo, D. & Kruisbeek, A. M. *Nature* **333**, 180-183 (1988).
7. Marrack, P. et al. *Cell* **53**, 627-634 (1988).
8. Blackman, M. A., Marrack, P. & Kappler, J. *Science* **244**, 214-217 (1989).
9. Liao, N.-S., Maltzman, J. & Raulet, D. H. *J. exp. Med.* **170**, 135-143 (1989).
10. Lo, D. & Sprent, J. *Nature* **319**, 672-675 (1986).
11. Zijlstra, M. et al. *Nature* **344**, 742-746 (1990).
12. Koller, B. H., Marrack, P., Kappler, J. W. & Smithies, O. *Science* **248**, 1227-1230 (1990).
13. Hoglund, P. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10332-10336 (1991).
14. Bernabeu, C., van de Rijn, M., Lerch, P. & Terhorst, C. *Nature* **308**, 642-645 (1984).
15. Allen H., Fraser, J., Flyer, D., Calvin, S. & Flavell, R. *Proc. natn. Acad. Sci. U.S.A.* **83**, 7447-7451 (1986).
16. von Boehmer, H. *Rev. Immun.* **8**, 531-556 (1990).
17. Longo, D. L., Kruisbeek, A. M., Davis, M. L. & Matis, L. A. *Proc. natn. Acad. Sci. U.S.A.* **82**, 5900-5904 (1985).
18. Huesmann, M., Scott, B., Kisielow, P. & von Boehmer, H. *Cell* **66**, 533-540 (1991).
19. van Ewijk, W. A. *Rev. Immun.* **9**, 591-615 (1991).
20. Gosgrove, D. G. et al. *Cell* **66**, 1051-1066 (1991).
21. Grusby, M., Johnson, R. S., Papaioannou, V. & Glimcher, L. H. *Science* **253**, 1417-1420 (1991).
22. Jenkins, M. K., Ashwell, J. D. & Schwartz, R. H. *J. Immun.* **140**, 3324-3330 (1988).
23. Speiser, D. E., Stubi, U. & Zinkernagel, R. M. *Nature* **355**, 170-172 (1992).
24. Bix, M. et al. *Nature* **349**, 329-331 (1991).
25. Raulet, D. H., Gottlieb, P. & Bevan, M. J. *J. Immun.* **125**, 1136-1143 (1980).
26. Lo, D., Ron, Y. & Sprent, J. *Immun. Res.* **5**, 221-232 (1986).
27. Bix, M. & Raulet, D. *J. exp. Med.* **176**, 829-834 (1992).

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1. Bevan, M. J. & Fink, P. *Immun. Rev.* **42**, 3-19 (1978).
2. Zinkernagel, R. M. et al. *J. exp. Med.* **147**, 882-896 (1978).
3. Sprent, J. *J. exp. Med.* **147**, 1159-1174 (1978).

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Elongation factor-1 α gene determines susceptibility to transformation

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ELONGATION factor-1 α (EF-1 α), an essential component of the eukaryotic translational apparatus, is a GTP-binding protein that catalyses the binding of aminoacyl-transfer RNAs to the ribosome¹⁻³. Expression of the EF-1 α gene decreases towards the end of the lifespans of mouse and human fibroblasts^{4,5}, but forced expression of EF-1 α prolongs the lifespan of *Drosophila melanogaster*⁶. Eukaryotic initiation factor-4E, another component of the translational machinery, is mitogenic or oncogenic when constitutively expressed in some mammalian cells⁷⁻⁹. Thus, components of the protein synthesis apparatus seem to be involved in the control of cell proliferation. Using expression cloning, we have isolated a complementary DNA clone from a BALB/c 3T3 mouse fibroblast variant, A31-I-13 (ref. 10), which specifies a factor determining the susceptibility of BALB/c 3T3 to chemically and physically induced transformation. Here we report that the factor is EF-1 α and that its constitutive expression causes BALB/c 3T3 A31-I-1 (ref. 10), C3H10T1/2 (ref. 11) and Syrian hamster SHOK¹² fibroblasts to become highly susceptible to transformation induced by 3-methylcholanthrene and ultraviolet light. EF-1 α messenger RNA is also constitutively expressed in a quiescent culture of the highly susceptible variant A31-I-13. We conclude that the removal of regulation of the expression of these components of the translational machinery may predispose cells to become more susceptible to malignant transformation.

BALB/c 3T3 A31 cells are poorly susceptible to transformation by chemical carcinogens and ultraviolet radiation, but yield

highly susceptible clonal variants. A31-I-13 is one such variant¹⁰. To identify a cellular factor that determines the susceptibility of cells to chemically or physically induced neoplastic transformation, we made a cDNA expression library with the simian virus 40 (SV40) early promoter-based pcD2 vector¹³ and messenger RNA prepared from a quiescent culture of A31-I-13 cells¹⁰. Using a less transformation-susceptible subclone of BALB/c 3T3 A31, A31-I-1 (ref. 10), as a recipient for expression cloning, we screened the cDNA library for a clone able to enhance transformation-susceptibility. Of 2,800 G418-resistant colonies generated by the electroporation method¹⁴, 21 formed dense colonies with about twice the saturation density of the parent A31-I-1 cells. These dense colonies was chosen as candidates as this phenotype was associated with A31-I-13 cells^{15,16}. After repeated subculturing, only three colonies retained stable morphological features that were typical of A31-I-13 cells¹⁶.

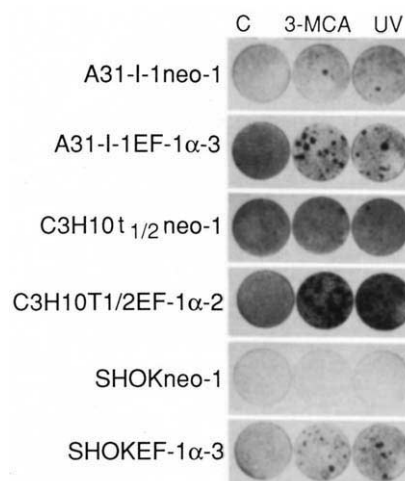


FIG. 1 Susceptibility to ultraviolet radiation- or methylcholanthrene-induced transformation of BALB/c 3T3 A31-I-1, C3H10T1/2 and SHOK cells transfected with an empty pcD2 vector or pcD2-EF-1 α . Cells were stained 5-7 weeks after exposure to 2.5 $\mu\text{g ml}^{-1}$ 3-methylcholanthrene (3-MCA) for 72 h or to 7.5 J m⁻² ultraviolet light (UV). C, unexposed control. Transformation was assayed by scoring transformed foci according to the criteria described previously^{11,12,17}. Transformed foci were confirmed by tumour production following subcutaneous injection into nude mice. For details, see Table 1.

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These were therefore examined for their transformation-susceptibility by using the 3-methylcholanthrene-induced transformation assay¹⁷. One colony was highly susceptible.

Genomic DNA was isolated from this colony and introduced into A31-I-1 cells to determine whether the ability to enhance the transformation-susceptibility was physically associated with the *neo* selectable marker gene, which is indicative of phenotypes induced by cDNA clones. In the second cycle of transfection, five stable G418-resistant colonies were obtained. Three of these were found to be highly susceptible to 3-methylcholanthrene-induced transformation. In a control experiment, in which an

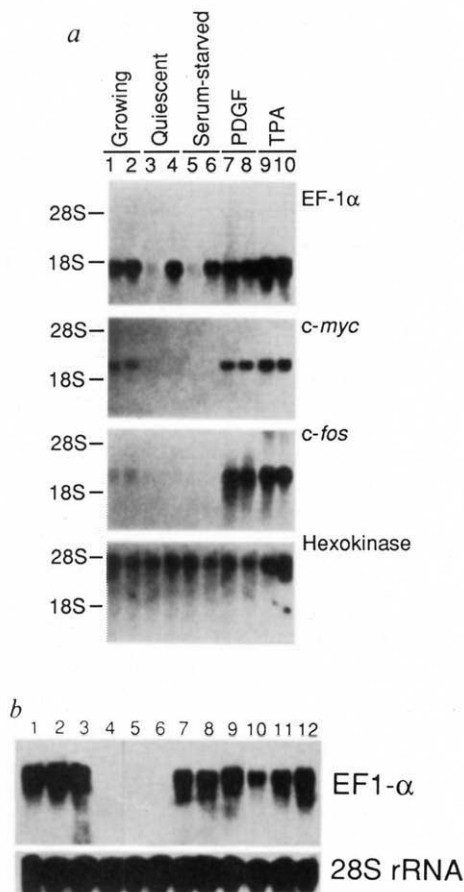


FIG. 2 *a*, Northern blot analysis of total RNA from exponentially growing (lanes 1 and 2) and density-arrested quiescent cells (lanes 3 and 4), and from serum-starved and TPA- or PDGF-treated cells (lanes 5–10), using the EF-1α cDNA as a probe. RNA from A31-I-1 cells are in lanes 1, 3, 5, 7 and 9; and RNA from A31-I-13 cells are in lanes 2, 4, 6, 8 and 10. The filters were reprobbed with *c-myc*²⁸, *c-fos*²⁹ and hexokinase cDNAs. For lanes 1–4, RNAs from actively growing cells (day 3) and density-arrested, quiescent cells (day 14) were used. To prepare RNA for lanes 5–10, density-arrested quiescent cells were incubated in serum-free medium for 24 h. Cells were then exposed for 30 min to fresh serum-free medium containing PDGF (40 ng ml⁻¹; lanes 7, 8) or TPA (100 ng ml⁻¹; lanes 9, 10). *b*, Northern blot analysis of total RNA from BALB/c 3T3 A31-I-1, C3H10T1/2 and SHOK cells transfected with an empty pcD2 vector or pcD2-EF-1α. RNA prepared from exponentially growing cells are in lanes 1–3 and 7–9; RNA from density-arrested quiescent cells are in lanes 4–6 and 10–12. Lanes 1 and 4, A31-I-1neo-1; lanes 2 and 5, C3H10T1/2neo-1; lanes 3 and 6, SHOKneo-1; lanes 7 and 10, A31-I-1EF-1α-3; lanes 8 and 11, C3H10T1/2EF-1α-2; lanes 9 and 12, SHOKEF-1α-3 (see Fig. 1). The hybridization probe was the EF-1α cDNA and the filter was rehybridized with an oligonucleotide probe for 28S RNA (lower panel).

METHODS. Cytoplasmic RNA was prepared by the guanidinium-caesium chloride method²³. Each lane contained 20 μg of total RNA. Blotting and hybridization were performed using standard protocols³⁰.

TABLE 1 Transformation-susceptibility and growth properties of cells lines constitutively expressing EF-1α

Cell line	Transformation frequencies ($\times 10^{-5} \pm \text{s.e.m.}$)		Growth properties	
	3-Methyl-cholanthrene	Ultraviolet radiation	Doubling time (h)	Saturation density ($\times 10^6$)
A31-I-1	21 $\pm$ 4.8	7 $\pm$ 0.2	16	1.5
A31-I-1neo-1	29 $\pm$ 5.3	14 $\pm$ 0.4	18	1.5
A31-I-1EF-1α-3	339 $\pm$ 36	118 $\pm$ 25	16	2.4
A31-I-1EF-1α-4	450 $\pm$ 43	197 $\pm$ 30	15	3.0
A31-I-1EF-1α-13	359 $\pm$ 44	156 $\pm$ 13	16	2.8
A31-I-1EF-1α-19	510 $\pm$ 33	194 $\pm$ 22	18	3.0
C3H10T1/2	9 $\pm$ 1.8	4 $\pm$ 2.6	19	0.8
C3H10T1/2neo-1	8 $\pm$ 2.5	5 $\pm$ 1.7	20	0.9
C3H10T1/2EF-1α-2	151 $\pm$ 12	119 $\pm$ 14	18	1.5
C3H10T1/2EF-1α-3	106 $\pm$ 12	82 $\pm$ 11	19	1.7
SHOK	<1.0	1 $\pm$ 1.0	15	2.4
SHOKneo-1	<1.0	1 $\pm$ 1.0	14	2.7
SHOKEF-1α-3	63 $\pm$ 16	63 $\pm$ 8.8	15	5.5
SHOKEF-1α-6	86 $\pm$ 15	98 $\pm$ 7.7	17	5.1

A31-I-1, C3H10T1/2 and SHOK cells were electroporated¹⁴ with pcD2-EF-1α or the empty pcD2 vector (neo) and G418-resistant colonies selected. The colonies were cultured to a density-arrested quiescent state, and the expression of EF-1α mRNA was examined by northern blot analysis. Colonies constitutively expressing EF-1α were chosen and used in further experimentation. None of the control colonies, obtained by transfection with an empty pcD2 vector, expressed EF-1α after growth arrest. The colonies constitutively expressing EF-1α were not morphologically transformed. A31-I-1EF-1α-4, C3H10T1/2EF-1α-2 and SHOKEF-1α-3 were examined for tumorigenicity by subcutaneous injection of 5×10^5 cells into nude mice, but none of them developed tumours even 50 days after inoculation. To determine transformation-susceptibility, 1×10^4 cells were exposed to 2.5 μg ml⁻¹ of 3-methylcholanthrene for 72 h or 7.5 J m⁻² of ultraviolet radiation, and were subsequently cultured for the formation of transformed foci. Transformed foci were judged according to the morphological criteria described previously^{11,12,17} and confirmed by tumour production after subcutaneous injection into nude mice. Transformation frequency was calculated from 8–10 dishes and expressed as a ratio of cells transformed to cells initially plated. To determine growth rates, 1×10^4 cells were plated into a 60-mm dish in triplicate and the cell number was counted every 48 h. Saturation density is the number of cells after culturing for 14 days.

equivalent cDNA library was constructed with mRNA from quiescent A31-I-1 cells and introduced back into A31-I-1 cells, we obtained three dense colonies out of 3,600 G418-resistant clones screened, but none of them were highly susceptible to transformation.

To isolate the cDNA responsible for inducing high transformation-susceptibility from the three candidate colonies, we took advantage of the presence of the SV40 replication origin in the pcD2 vector. Each of these three clonal cells were fused with COS cells¹⁸ to release the cDNA plasmids integrated into the host genome, which could then be recovered in *Escherichia coli*. Similar cDNA clones having a 1.7-kilobase insert were recovered from all three fused cells. They yielded the same restriction fragments when cleaved with *HindIII*, *PvuI*, *PvuII* or *SspI*, showing that they are indeed the same. Sequencing of one of the clones revealed that it contained a 56-base-pair (bp) 5' noncoding sequence and a 1,386-bp open reading frame followed by a 285-bp 3' noncoding sequence. This cDNA was slightly longer than the EF-1α cDNA isolated from Ehrlich ascites cells and, despite one mismatch in codon 399, identical in sequence¹⁹. We therefore designated it pcD2-EF-1α. Whereas the reported sequence of bases was GCA, repeated sequencing confirmed that our cDNA contained bases GCC. Nevertheless, both codons encode alanine, hence both cDNAs encode the same protein. This one-base mismatch may be due to sequence polymorphism. Transformation susceptibility is indeed

enhanced by pcD2-EF-1 α . The pcD2-EF-1 α was transfected into BALB/c 3T3 A31-I-1 (ref. 10) C3H10T1/2 (ref. 11) and SHOK¹² cells, and several independent clones constitutively expressing the EF-1 α cDNA were tested. Colonies of BALB/c 3T3 A31-I-1, C3H10T1/2 and SHOK showed a 10–20-fold increase in susceptibility to cell transformation induced by 3-methylcholanthrene or ultraviolet light compared with either their parent cell lines or those transfected with an empty vector (Table 1; Fig. 1). The constitutive expression of the EF-1 α cDNA did not alter growth rate, but caused the cells to grow to about twice the density of A31-I-1 cells (Table 1). These phenotypes are strikingly similar to that of the highly transformation-susceptible A31-I-13 cells, from which this cDNA is cloned^{15,16}.

The expression of the EF-1 α gene is tightly regulated in BALB/c 3T3 A31, its subclone A31-I-1, C3H10T1/2 and SHOK cells. Expression is switched off when growth is arrested, but is highly induced upon growth stimulation or during rapid growth (Fig. 2a and b). The level of induction was similar among the three cell lines. But in A31-I-13 cells, the EF-1 α gene was not switched off after becoming quiescent, and was expressed constitutively at a level comparable to that in rapidly growing cells (Fig. 2a, lanes 4 and 6). Similarly, in pcD2-EF-1 α -transfected A31-I-1, C3H10T1/2 and SHOK cells which became hypersensitive to ultraviolet radiation and methylcholanthrene (Fig. 1; Table 1), EF-1 α mRNA continued to be expressed after quiescence (Fig. 2b, lanes 10–12). On the basis of these results, we conclude that constitutive expression of EF-1 α is the main, if not sole, factor determining this hypersensitivity of A31-I-13 cells to transformation. The mechanism for the constitutive expression of EF-1 α in A31-I-13 is unclear, but comparative Southern blot analysis of A31-I-1 and A31-I-13 cells has so far revealed no apparent structural alterations in the EF-1 α gene (data not shown).

Quiescent BALB/c 3T3 A31 and its subclone A31-I-1 require two types of growth factor to resume DNA synthesis²⁰. One is called a 'competence factor' and may be platelet-derived growth factor (PDGF) or a phorbol ester such as 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). The other is called a 'progression

factor' and may be epidermal growth factor (EGF) or insulin. Interestingly, quiescent A31-I-13 cells are already competent and therefore require only a progression factor to enter S phase²⁰ (Fig. 3). PDGF itself has a transformation function, but TPA increases the frequency of chemically or physically induced transformation of A31-I-1 cells²¹. We therefore investigated whether EF-1 α gene expression could be modulated by PDGF and TPA treatments and, if so, whether it might mediate the growth competence induced by these treatments. Both treatments induced EF-1 α gene expression in quiescent A31-I-1 cells, although TPA was more potent (Fig. 2a, lanes 7–10). Moreover, A31-I-1 cells constitutively expressing the EF-1 α cDNA were already competent, requiring only a progression factor to resume DNA synthesis (Fig. 3). We also investigated the levels of c-Myc and c-Fos mRNAs (these messages being generally associated with growth stimulation) in A31-I-1, A31-I-13 and A31-I-1 cells treated with PDGF or TPA. Levels were elevated only in the PDGF- or TPA-treated cells (Fig. 2a, lanes 7–10). We conclude that the competency for DNA synthesis induced by TPA and PDGF is mediated by EF-1 α gene expression, but not by c-Myc or c-Fos expression.

Although the exact mechanism by which constitutive expression of EF-1 α enhances the susceptibility to chemically induced or radiation-induced transformation is unclear, it is likely that EF-1 α functions as a tumour promoter by making cells competent for growth rather than hypersensitive to ultraviolet light or carcinogens. A31-I-13 cells are neither hypermutable nor hypersensitive to carcinogens^{10,22}, but both A31-I-13 itself and A31-I-1 constitutively expressing EF-1 α are competent for growth (Fig. 3). How EF-1 α makes cells competent for growth is also unclear. EF-1 α may serve as a downstream component of growth signalling pathways. The *v-fos* gene induces the expression of EF-1 α ²³, and during the G0–G1 transition actin organization undergoes drastic changes^{24,25}. Apart from its role as an essential factor in translation, EF-1 α may also be involved in this process through its actin-binding activity²⁶. In fact, A31-I-13 cells display an alteration in the organization of actin filaments¹⁶. Such an alteration might be required for cells to respond to a progression factor to initiate DNA replication. Alternatively, constitutive expression of EF-1 α may make cells growth-competent simply by increasing the rate of translation of genes essential for growth. Quiescent A31-I-13 cells are indeed 70–80% more active in protein synthesis than quiescent A31-I-1 cells (data not shown).

Our findings that the translational factor, EF-1 α , renders BALB/c 3T3, C3H10T1/2 and SHOK cells highly susceptible to chemically or physically induced neoplastic transformation and that it concomitantly makes cells competent for DNA synthesis are very important in the understanding of multistage carcinogenesis. As most post-embryonic cell proliferation is controlled at both the G0–G1 and G1–S transitions²⁷, any event that allows cells to progress into S phase must be a step towards the uncontrolled cell growth characteristic of malignant transformation. □

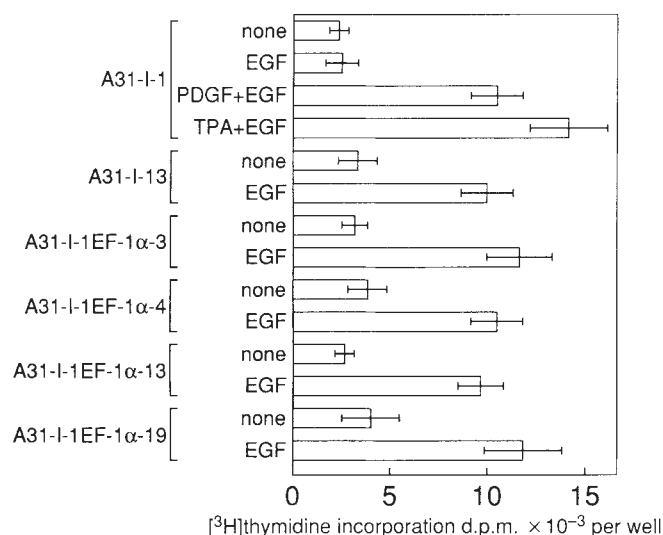


FIG. 3 Responses of A31-I-1, A31-I-13 and EF-1 α cDNA-transfected A31-I-1 to competence and progression factors. Induction of DNA synthesis in response to exogenous factors was assayed. Density-arrested, serum-starved cells were incubated for 24 h with EGF (100 ng ml⁻¹), PDGF (40 ng ml⁻¹) and/or TPA (100 ng ml⁻¹) in 0.2 ml of medium containing 1 μ Ci ml⁻¹ or [³H]thymidine (20 Ci mmol⁻¹). The amount of [³H]thymidine incorporated was determined as described previously²⁰. The results shown are averaged numbers for triplicate experiments. Bars indicate standard deviations.

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- Karri, T. *Biochim. biophys. Acta* **505**, 95–127 (1978).
- Hershey, J. W. B. *Cell Biology, a Comprehensive Treatise* Vol. 4 (eds Goldstein, L. & Prescott, D. N.) 1–68 (Academic, New York, 1980).
- Moldave, K. A. *Rev. Biochem.* **54**, 1109–1119 (1985).
- Webster, G. C. *Molecular Biology of Aging, Gene Stability and Gene Expression* (eds Sohal, R. S., Birnbaum, L. S. & Culter, R. G.) 263–289 (Raven, New York, 1985).
- Cavallius, J., Rattan, S. I. S. & Clark, B. F. C. *Expl. Gerontol.* **21**, 149–157 (1986).
- Shepherd, J. C. W., Walldorf, U., Hug, P. & Gehring, W. J. *Proc. natn. Acad. Sci. U.S.A.* **86**, 7520–7521 (1989).
- Lazaris-Karatzas, A., Montine, K. S. & Sonenberg, N. *Nature* **345**, 544–547 (1990).
- Smith, M. R. et al. *New Biol.* **2**, 648–654 (1990).
- Benedetti, A. D. & Rhoads, R. E. *Proc. natn. Acad. Sci. U.S.A.* **87**, 8212–8216 (1990).
- Kakunaga, T. & Crow, J. D. *Science* **209**, 505–507 (1980).
- Reznikoff, C. A., Brankow, D. W. & Heidelberger, C. *Cancer Res.* **33**, 3231–3238 (1973).
- Higashi, T., Sasai, H., Suzuki, F., Miyoshi, J. & Kakunaga, T. *Proc. natn. Acad. Sci. U.S.A.* **87**, 2409–2413 (1990).
- Chem, C. & Okayama, H. *Molec. cell Biol.* **7**, 2745–2752 (1987).
- Tatsuka, M., Orita, S., Yagi, T. & Kakunaga, T. *Expl. Cell Res.* **178**, 154–162 (1988).

15. Yamasaki, H., Enomoto, T., Shiba, Y., Kanno, Y. & Kakunaga, T. *Cancer Res.* **45**, 637–641 (1985).
16. Tataka, M., Owada, M. K. & Mitsui, H. *Cancer Res.* **52**, 4232–4241 (1992).
17. Kakunaga, T. in *IARC Scientific Publication No. 67: Transformation Assay of Established Cell Lines, Mechanisms and Application* (eds Kakunaga, T. & Yamasaki, H.) 55–73 (Oxford University Press, New York, 1985).
18. Breitman, M. L., Tsui, L. C., Buckwald, M. & Siminovich, L. *Molec. cell. Biol.* **2**, 966–976 (1982).
19. Lu, X. & Werner, D. *Nucleic Acids Res.* **17**, 442 (1989).
20. Tatsuka, M., Orita, S. & Kakunaga, T. *J. cell. Physiol.* **139**, 18–23 (1989).
21. Hirakawa, T., Kakunaga, T., Fujiki, H. & Sugimura, T. *Science* **216**, 527–529 (1982).
22. Lo, K.-Y. & Kakunaga, T. *Cancer Res.* **42**, 2644–2650 (1982).
23. Taniguchi, S., Miyamoto, S., Sadano, H. & Kobayashi, H. *Nucleic Acids Res.* **19**, 6949 (1991).
24. Bockus, B. J. & Stiles, C. D. *Exp. Cell Res.* **153**, 186–197 (1984).
25. Herman, B. & Pledger, W. J. *J. Cell Biol.* **100**, 1031–1040 (1985).
26. Yang, F., Demma, M., Warren, V., Dharmawardhane, S. & Condeelis, J. *Nature* **347**, 494–496 (1990).
27. Pardee, A. B. *Science* **246**, 603–608 (1989).
28. Yagi, T., Sasayama, S., Sasai, H. & Kakunaga, T. *Molec. Carcinogenesis* **1**, 222–228 (1989).
29. Curran, T., Peters, G., Beveren, C. V., Teich, N. M. & Verma, I. M. *J. Virol.* **44**, 672–678 (1982).
30. Ausabel, F. M. et al. *Current Protocols in Molecular Biology* (Wiley, New York, 1987).

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Cell transformation and activation of pp60^{c-src} by overexpression of a protein tyrosine phosphatase

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THE kinase activity of pp60^{c-src} is specifically and transiently increased during mitosis and repressed during interphase¹. Loss of cell-cycle control of pp60^{c-src} occurs on mutation of Tyr 527 to Phe or when pp60^{c-src} is associated with polyoma middle-T-antigen, and these conditions result in cell transformation or tumorigenesis^{2,3}. In both cases, pp60^{c-src} has elevated kinase activity which is maintained throughout the cell cycle and accompanied by dephosphorylation of the carboxy-terminal negative regulatory^{4–7} Tyr 527 site, or mimicry of Tyr 527 dephosphorylation in the case of the mutant. Here we report that overexpression of the receptor-like protein tyrosine phosphatase PTPα^{8–10} results in persistent activation of pp60^{c-src} kinase, with concomitant cell transformation and tumorigenesis. In PTPα-overexpressing cells, the pp60^{c-src} kinase activation is accompanied by dephosphorylation at Tyr 527, and direct dephosphorylation of this site by purified PTPα occurs *in vitro*. Our results suggest that PTPα is involved in the regulation of cell proliferation, exerting at least some of its effects through pp60^{c-src} kinase, and has oncogenic capability when overexpressed.

Fischer rat embryo fibroblasts (REF) were transfected with the full-length human receptor-like protein tyrosine phosphatase-α complementary DNA in an expression vector driven by the human cytomegalovirus promoter and G418-resistant cell lines were isolated. Western blotting with anti-PTPα antibodies detected elevated levels of a protein of an expected¹⁰ *M_r* ~130K in total lysates of several cell lines (not shown), and three lines (α-7, α-21, α-29) were selected for further analysis. In accord with the transmembrane nature of PTPα, solubilized membrane fractions from these cells contained much higher amounts of PTPα than those from parental cells (REF), and the overexpressed PTPα was predominantly localized to the membrane, although some was detectable in the cytosolic fraction (Fig. 1a). The membrane PTPase specific activities were elevated threefold over those in parental and control cells transfected with expression plasmid lacking PTPα cDNA (REF-neo), whereas cytosolic PTPase specific activities were increased only 1.5-fold (Fig. 1b). Immunoprecipitates of PTPα from lysates of ³⁵S-labelled α-7, α-21 and α-29 cells contained a prominent labelled band on analysis by SDS-polyacrylamide gel electrophoresis

(PAGE) corresponding to the expected size of PTPα (data not shown). Immunoprecipitates of PTPα from α-7, α-21 and α-29 cell lysates also had higher phosphatase activity than those prepared from control or parental cell lysates (Fig. 1c), demonstrating that the observed increases in PTPase specific activities are due to PTPα overexpression.

At subconfluency, the PTPα-overexpressing cells had a fibroblastic phenotype (Fig. 2a). At this stage the α-21 cells were beginning to exhibit a transformed phenotype with some of the cells appearing rounded and highly refractile. In contrast to the control cells, none of the PTPα-overexpressing cell lines were growth-arrested through contact inhibition (Table 1). They grew in multilayers and formed foci containing refractile and disordered cells (Fig. 2b). The α-7, α-21 and α-29 cells also had a reduced requirement for serum growth factors as indicated by their continued proliferation in medium with 0.1% fetal calf serum (FCS), whereas control cells became quiescent (Table 1). In particular, a high proportion of α-21 cells appeared to be transformed within 24 h of serum deprivation (Fig. 2c). The α-7 and α-29 cells also appeared to be transformed after the concentration of FCS in the medium was reduced from 10% to 0.1%, although this phenotypic change was more apparent after 48–72 h (Fig. 2d). All three PTPα-overexpressing cells exhibited anchorage-independent growth in soft agar (with α-7 and α-21 cells showing enhanced colony formation in the absence of serum) (Fig. 2e, and Table 1) and the ability to form tumours in nude mice with a short latency period (Table 1). The above features of these cells in culture in conjunction with their demonstrated *in vivo* tumorigenicity indicates that PTPα has transforming capability and may be oncogenic when overexpressed.

A few enzymes are activated by tyrosine dephosphorylation, such as the *src* family tyrosine kinases^{4,11–13} and the cell cycle serine/threonine kinase p34^{cdc2} (refs 14–16). Of these, certain mutant forms of pp60^{c-src}, including its oncogenic counterpart pp60^{v-src}, have transforming ability^{5–7,17}. The activity of pp60^{c-src} in the control REF-neo and PTPα-overexpressing cells was tested by immunoprecipitating pp60^{c-src} and measuring its ability to phosphorylate the exogenous substrate enolase. In cells maintained in medium with 0.1% FCS for 48 h, the kinase activity of pp60^{c-src} from α-7, α-21 and α-29 cells was increased three- to sixfold over that of pp60^{c-src} from control cells, and the autophosphorylation activity of pp60^{c-src} was also higher (Fig. 3a, top). A similar activation of pp60^{c-src} was observed in cells maintained as above but treated with 10% FCS for 8 h (Fig. 3a, top) or 24 h (not shown). The amounts of pp60^{c-src} protein immunoprecipitated from all cells were comparable, as determined by probing immunoblots with an anti-*src* protein monoclonal antibody (Fig. 3a, bottom). Thus the higher activity of pp60^{c-src} in the PTPα-overexpressing cells is not due to a corresponding increase in the level of pp60^{c-src} protein.

In many instances, activation of pp60^{c-src} involves dephosphorylation of Tyr 527^{4–7,18}. To determine whether the activation of pp60^{c-src} in PTPα-overexpressing cells could be accounted for by dephosphorylation of this residue, we immunoprecipitated pp60^{c-src} from ³²P-labelled cells and analysed its cyanogen bromide phosphopeptides. A 32K phosphopeptide containing amino terminal sites for serine and threonine phosphorylation^{19,20} was recovered from cleaved pp60^{c-src} from both control (REF-neo) and PTPα-overexpressing cells (Fig. 3b, open arrow). But the pp60^{c-src} from control REF-neo cells was phosphorylated at Tyr 527 (represented by a 4K phosphopeptide¹⁹), whereas no phosphorylation of Tyr 527 was detected in pp60^{c-src} from α-7, α-21 and α-29 cells (Fig. 3b, closed arrow). A direct interaction between PTPα and pp60^{c-src} could be demonstrated *in vitro*, where bacterially expressed and purified PTPα could dephosphorylate Tyr 527 of pp60^{c-src} immunoprecipitated from quiescent control cells (Fig. 3c). Although Tyr 416 is usually phosphorylated when Tyr 527 is not phosphorylated^{5–7}, no phosphorylation at Tyr 416 (which would be represented by a 9K phosphopeptide¹⁹) was observed in pp60^{c-src} from α-7, α-21 and

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TABLE 1 Growth characteristics of parental, control and PTP α -overexpressing cells

Cells	Saturation density* (cells cm ⁻²)	Cell proliferation at 0.1% FCS	Colonies in soft agar†		Tumours in nude mice (latency)‡
			10% FCS	0% FCS	
REF	ND	No	0	0	0/5
REF-neo	1.2×10^5	No	0	0	0/4
REF α -7	$>1.5 \times 10^6$	Yes	217 ± 20	416 ± 11	4/4 (5-6d)
REF α -21	$>1.5 \times 10^6$	Yes	420 ± 18	789 ± 42	4/4 (5-6d)
REF α -29	$>1.5 \times 10^6$	Yes	781 ± 1	456 ± 28	4/4 (5-6d)

ND, not determined.

* No apparent saturation density was observed with α -7, α -21 or α -29 cells. Proliferation continued to the point where fresh medium was required every 3-5 h and eventually these cells lifted off the dishes in sheets.

† 400 cells were added to each 6-cm Petri dish and colonies were counted after 3 weeks. Counts are the average of triplicate experiments $\pm$ standard error.

‡ Each mouse was injected subcutaneously at one site on the back with 2×10^5 cells.

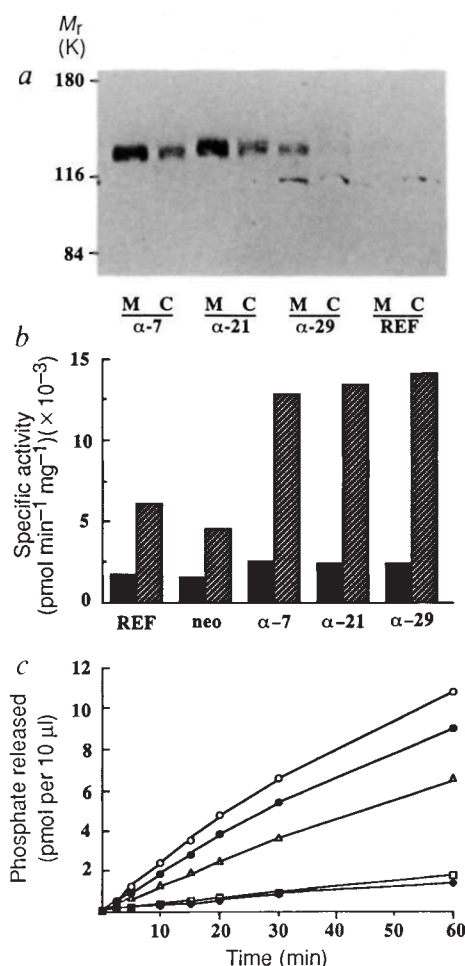
α -29 cells (Fig. 3b). This suggests that Tyr 416 and Tyr 527 do not undergo coordinated inverse phosphorylation and dephosphorylation cycles, or more likely, that PTP α dephosphorylates Tyr 416 as rapidly as this site is phosphorylated.

The activation state of pp60^{c-src} reflects the balance of oppos-

ing activities of a Tyr 527 kinase and phosphatase, where phosphorylation of Tyr 527 negatively regulates pp60^{c-src} activity. A specific Tyr 527 kinase has recently been identified and cloned^{21,22}, although as yet nothing is known of the regulation of its activity. Cell cycle-regulated inhibition of such a kinase

FIG. 1 Overexpression of active PTP α . a, Western blotting with anti-PTP α antiserum of solubilized membrane (M) and cytosolic (C) fractions from parental cells (REF) and cells transfected with PTP α cDNA (α -7, α -21, α -29). b, Total PTPase specific activities of cytosolic (solid bars) and solubilized membrane (hatched bars) fractions measured towards the phosphotyrosyl-peptide RR-src. Fractions from control-transfected cells (REF-neo) are designated as neo. c, PTPase activity of immunoprecipitated PTP α from parental REF ($\square$), control transfected REF-neo ($\blacklozenge$) and PTP α -transfected ($\bullet$, α -7; $\circ$, α -21; Δ , α -29) cells.

METHODS. To aid cloning of the full-length PTP α cDNA²⁷ into the *Bam*H1-*Hind*III site of the expression vector pXJ41 (ref. 28), a 570 bp fragment of the 5' portion of the gene was first amplified by polymerase chain reaction (PCR). A *Bam*H1 site (GGATCC) and an optimal Kozak consensus sequence (CCACC) were added to the 5' oligonucleotide (5'-AAGGATCCAAACCAC-CATGGATTCTGG-3') and the 3' oligonucleotide contained the unique *Ecl*XI restriction site in the PTP α cDNA. The fidelity of amplification was confirmed by sequencing. The PCR fragment was cleaved with *Bam*H1 and *Ecl*XI and ligated with a *Ecl*XI-*Hind*III fragment containing the rest of the PTP α coding sequence, and this was inserted into a *Bam*H1-*Hind*III site in the expression vector pXJ41 to give pXJ41-PTP α . The neo gene in a pMAM-neo vector (Pharmacia) was excised with *Bam*H1 and blunt-ended by filling with Klenow. It was inserted into the pXJ41-PTP α and pXJ41 vectors which had been cut at a *Sal*I site (downstream of the PTP α insert in the former) and likewise blunt-ended. The REF cells ($\sim 10^6$ per 15-cm dish) were transfected with 20 μ g of either pXJ41-PTP α -neo or pXJ41-neo expression vector using the calcium phosphate method²⁹. After 16 h the cells were washed with DME medium (containing 4.5 g l⁻¹ glucose and penicillin/streptomycin) and incubated with DME medium containing G418 (500 μ g ml⁻¹) and 10% FCS. The medium was changed every 4 days, and colonies were isolated after 21 days. To prepare cell membrane and cytosolic fractions, confluent cells were rinsed twice with cold PBS, scraped into collecting buffer (50 mM Tris-Cl pH 7.6, 150 mM NaCl, 2 mM PMSF, 10 μ g ml⁻¹ aprotinin) and pelleted by low-speed centrifugation. After resuspension of the pellets in collecting buffer and brief sonication to lyse the cells, the lysates were centrifuged at 100,000g for 30 min at 4 °C to obtain the cytosolic supernatants. The pellets were rinsed once with collecting buffer, resuspended in collecting buffer with 1% Triton X-100 and solubilized on ice for 60 min. After centrifugation at 100,000g as above the supernatants comprised the solubilized membrane fractions. For western blotting, 150 μ g protein from each fraction was electrophoresed on 7.5% SDS-PAGE and transferred to nitrocellulose membranes in 50 mM Tris base, 0.56% glycine, 10% methanol (80 V, 75 min, 25 °C). The membranes were probed with a 1:500 dilution of anti-PTP α antiserum (raised in rabbits against a peptide comprising the C-terminal amino-acid sequence IDAFSDYANFK of PTP α) followed by a 1:3,000 dilution of goat anti-rabbit IgG conjugated to peroxidase (Sigma), and developed using the ECL system (Amersham). PTPase specific activity was measured at 30 °C in reactions containing 50 mM Mes pH 6.0, 0.5 mg ml⁻¹ BSA, 0.5 mM DTT, 5 μ M phosphotyrosyl-RR-src peptide (RRLLDAEY(P)AARG, corresponding to the sequence encompassing Tyr 416 of pp60^{c-src} and phosphorylated as described²⁷) and 3.3 μ g ml⁻¹ solubilized membrane protein or 6.6



μ g ml⁻¹ cytosolic protein. Reactions were linear over the times assayed. For immunoprecipitation of PTP α , confluent cells were lysed in collecting buffer with 1% Triton X-100 and centrifuged at 100,000g for 30 min. Anti-PTP α antiserum (150 μ l) was added to 6 ml of 2 mg ml⁻¹ supernatant and mixed at 4 °C for 16 h. Protein A-Sepharose (Pharmacia) was added and mixed at 4 °C for 2 h. The immunoprecipitated pellets obtained by centrifugation were washed three times with 50 mM Tris-Cl pH 7.6, 0.5 M NaCl, 1 mM PMSF, 10 μ g ml⁻¹ aprotinin and packed into small columns in the same buffer. Protein was eluted from the beads with 0.6 ml 0.1 M glycine, pH 2.5 and immediately neutralized with 20 μ l 1 M Tris-Cl, pH 8.0.

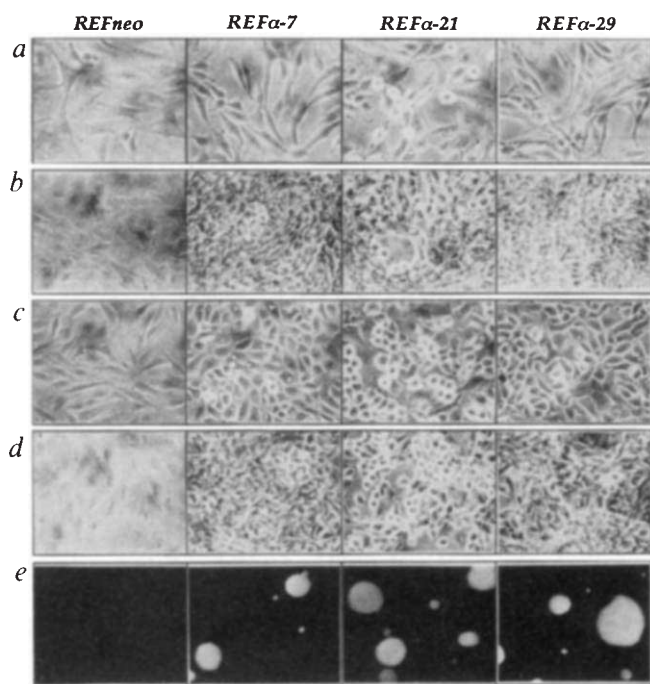


FIG. 2 Morphologies of control REF-neo and PTP α -overexpressing cells. *a*, Subconfluent and *b*, 4-day postconfluent cells in DME medium with 10% FCS ($\times 320$). *c*, 24 h after changing to DME medium with 0.1% FCS ($\times 320$). *d*, 72 h after changing to DME medium with 0.1% FCS ($\times 320$). *e*, Colonies on soft agar with serum-free medium ($\times 32$).

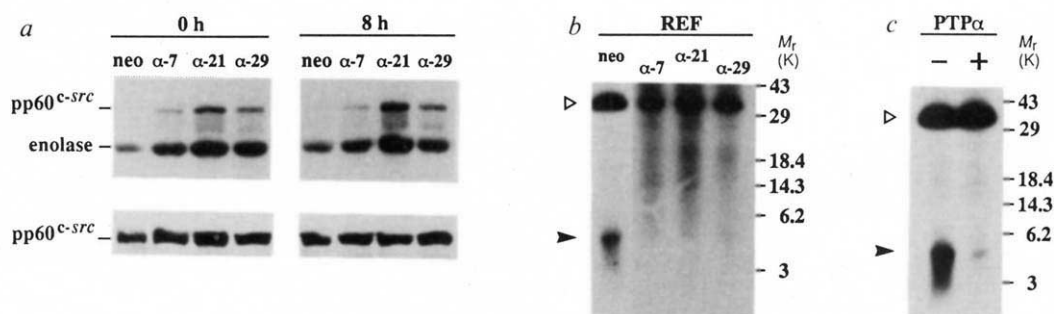
or activation of an appropriate tyrosine phosphatase, or both, could result in Tyr 527 dephosphorylation and activation of mitotic pp60^{c-src}. The unregulated occurrence of these events may lead to uncontrolled cell proliferation. In accord with this, we have shown that the constitutive overexpression of active PTP α subverts the normal control of cell growth and have

identified pp60^{c-src} as a target for PTP α action. The *in vivo* dephosphorylation of Tyr 527 of pp60^{c-src} in PTP α -overexpressing cells and the cellular association of both proteins with membranes²³, when coupled with the ability of purified PTP α to dephosphorylate the same site of pp60^{c-src} *in vitro*, suggests that pp60^{c-src} may be a direct substrate of PTP α . Because PTP α is widely expressed and is particularly abundant in the brain^{9,10} whereas pp60^{c-src} is expressed in most cells and at high levels in neural tissue²⁴, PTP α may function as an *in vivo* regulator of *src* kinase activity in normal cells. In view of the mitosis-specific activation of pp60^{c-src}, we are investigating the activity of PTP α at different stages of the cell cycle.

The PTPases have been considered as potential anti-oncogenes^{25,26}, where mutations giving rise to a nonfunctional

FIG. 3 Protein kinase activity, immunoblotting and cyanogen bromide phosphopeptide analysis of pp60^{c-src}. Cells maintained in medium with 0.1% FCS for 48 h were collected (0 h) or treated with 10% FCS for 8 h before collection (8 h). Lysates of control REF-neo cells (neo) and PTP α -overexpressing (α -7, α -21, α -29) cells were equalized for protein and immunoprecipitated with anti-*src* monoclonal antibody 327. *a*, Top, a portion of each immunoprecipitate was incubated with the exogenous substrate enolase and [γ -³²P]ATP and the mixture resolved on 10% SDS-PAGE followed by autoradiography. Bottom, another portion of each immunoprecipitate was run on 10% SDS-PAGE, immunoblotted and probed with antibody 327 to measure pp60^{c-src} levels. *b*, Cyanogen bromide phosphopeptides of immunoprecipitated pp60^{c-src} from lysates of cells maintained in medium with 0.1% FCS for 48 h and then metabolically labelled with ³²P-orthophosphate for 16 h. *c*, As in *b* except that pp60^{c-src} immunoprecipitated from quiescent control REF-neo cells was incubated without (minus) or with (plus) the bacterially expressed and purified intracellular region of PTP α before digestion with cyanogen bromide. In *b* and *c*, the position of the 32K phosphopeptide is designated by the open arrow and the position of the 4K phosphopeptide containing Tyr 527 is designated by the closed arrow.

METHODS. Cells were lysed in RIPA buffer³⁰ (2 ml per 15-cm dish) and the lysates centrifuged at 100,000*g* for 30 min. Anti-*Src* monoclonal antibody (mAb 327, Oncogene Science) was added to the lysates (1 μ g per 2 ml) and mixed at 4 °C for 1 h. Goat anti-mouse IgG (Sigma) was added (4 μ g per 2 ml) and mixed as above, followed by a one-tenth volume of protein A cell suspension (Sigma) and mixing as above. After low-speed centrifugation the immunoprecipitates were washed as described³⁰. Immunoblot analysis of immunoprecipitated pp60^{c-src} levels was as described in Fig. 1, except



immunoblots were probed with a 1:1,000 dilution of 0.1 mg ml⁻¹ mAb 327 followed by goat anti-mouse IgG conjugated to peroxidase (1:1,000) (Sigma). The kinase activity of immunoprecipitated pp60^{c-src} was determined in 20 μ l reactions containing 10 mM PIPES, pH 7.0, 5 mM MnCl₂, 0.5 mM DTT, 2.5 μ g acid-treated enolase and 10 μ Ci [γ -³²P]ATP at 37 °C for 5 min. For cyanogen bromide phosphopeptide analysis, cells were labelled for 16 h with 1.5 mCi ml⁻¹ ³²P-orthophosphate (Amersham) in phosphate-free DME containing normal DME (95/5, v/v) and 0.1% FCS. Immunoprecipitates of pp60^{c-src}, prepared from the various cells as just described, were electrophoresed on 10% SDS-PAGE and the position of ³²P-labelled pp60^{c-src} determined by autoradiography. Corresponding pieces of the gel were cut out and electroeluted in 25 mM Tris, 192 mM glycine, 0.1% SDS, 2% 2-mercaptoethanol at 90 V for 2 h. Electroeluted protein was precipitated with 10% trichloroacetic acid with 100 μ g BSA as carrier. The precipitate was washed once with cold ethanol, dried, digested with cyanogen bromide and analysed on 20% SDS-PAGE as described¹⁹. For *in vitro* treatment of pp60^{c-src} with PTP α before cyanogen bromide digestion, ³²P-labelled pp60^{c-src} immunoprecipitates were washed four times with 50 mM Mes, pH 6.0, 0.5 mM DTT and incubated at 30 °C for 60 min in the same buffer with 0.5 μ g ml⁻¹ BSA in the presence or absence of 15 μ g PTP α (intracellular domain)²⁷.

gene product or gene deletion could result in tumorigenesis. The transforming ability of overexpressed PTP α indicates that certain PTPases may instead function as oncogenes, perhaps when overexpressed as a consequence of gene translocation or amplification, or of other defects affecting normal regulatory mechanisms. □

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1. Chackalaparampil, I. & Shalloway, D. *Cell* **52**, 801–810 (1988).
2. Bagrodia, S., Chackalaparampil, I., Kmiecik, T. E. & Shalloway, D. *Nature* **349**, 172–175 (1991).
3. Kaech, S., Covik, L., Wyss, A. & Ballmer-Hofer, K. *Nature* **350**, 431–433 (1991).
4. Cooper, J. A., Gould, K. L., Cartwright, C. A. & Hunter, T. *Science* **231**, 1431–1434 (1986).
5. Kmiecik, T. E. & Shalloway, D. *Cell* **49**, 65–73 (1987).
6. Plwnica-Worms, H., Saunders, K. B., Roberts, T. M., Smith, A. E. & Cheng, S. H. *Cell* **49**, 75–82 (1987).
7. Cartwright, C. A., Eckhart, W., Simon, S. & Kaplan, P. L. *Cell* **49**, 83–91 (1987).
8. Grueger, N. X., Streuli, M. & Saito, H. *EMBO J.* **9**, 3241–3252 (1990).
9. Matthews, R. J., Cahir, E. D. & Thomas, M. L. *Proc. natn. Acad. Sci. U.S.A.* **87**, 4444–4448 (1990).
10. Sap, J., D'Eustachio, P., Givol, D. & Schlessinger, J. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6112–6116 (1990).

11. Marth, J. D. *et al. Molec. cell Biol.* **8**, 540–550 (1988).
12. Amrein, K. E. & Sefton, B. M. *Proc. natn. Acad. Sci. U.S.A.* **85**, 4247–4251 (1988).
13. Kawakami, T., Kawakami, Y., Aaronson, S. A. & Robbins, K. C. *Proc. natn. Acad. Sci. U.S.A.* **85**, 3870–3874 (1988).
14. Dunphy, W. G. & Newport, J. W. *Cell* **58**, 181–191 (1989).
15. Morla, A. O., Draetta, G., Beach, D. & Wang, J. Y. *J. Cell Biol.* **58**, 193–203 (1989).
16. Gould, K. L. & Nurse, P. *Nature* **342**, 39–45 (1989).
17. Parker, R. C., Varmus, H. E. & Bishop, J. M. *Cell* **37**, 131–139 (1984).
18. Courtneige, S. A. *EMBO J.* **4**, 1471–1477 (1985).
19. Bolen, J. B., Veillette, A., Schwartz, A. M., DeSeau, V. & Rosen, N. *Oncogene Res.* **1**, 149–168 (1987).
20. Shenoy, S. *et al. Cell* **57**, 763–774 (1989).
21. Nada, S., Okada, M., MacAuley, A., Cooper, J. A. & Nakagawa, H. *Nature* **351**, 69–72 (1991).
22. Okada, M. & Nakagawa, H. *J. Biol. Chem.* **264**, 20886–20893 (1989).
23. Courtneige, S. A., Levinson, A. D. & Bishop, J. M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3783–3787 (1980).
24. Cotton, P. C. & Brugge, J. S. *Molec. cell Biol.* **3**, 1157–1162 (1983).
25. Fischer, E. H., Charbonneau, H. & Tonks, N. K. *Science* **253**, 401–406 (1991).
26. La Forgia, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 5036–5040 (1991).
27. Wang, Y. & Pallen, C. J. *EMBO J.* **10**, 3231–3237 (1991).
28. Xiao, J. H., Davidson, I., Matthes, H., Garner, J. M. & Chambon, P. *Cell* **65**, 551–568 (1991).
29. Graham, F. L. & Van Der Eb, J. *Virology* **52**, 456–467 (1973).
30. Gould, K. L. & Hunter, T. *Molec. cell Biol.* **8**, 3345–3356 (1988).

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The candidate oncoprotein Bcl-3 is an antagonist of p50/NF- κ B-mediated inhibition

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THE candidate oncogene *bcl-3* was discovered as a translocation into the immunoglobulin alpha-locus in some cases of B-cell chronic lymphocytic leukaemias¹. The protein Bcl-3 contains seven so-called ankyrin repeats. Similar repeat motifs are found in a number of diverse regulatory proteins but the motifs of Bcl-3 are most closely related to those found in I κ B proteins in which the ankyrin repeat domain is thought to be directly involved in inhibition of NF- κ B activity. No biological function has yet been described for Bcl-3, but it was noted recently² that Bcl-3 interferes with DNA-binding of the p50 subunit of NF- κ B *in vitro*. Here we demonstrate that Bcl-3 can aid κ B site-dependent transcription *in vivo* by counteracting the inhibitory effects of p50/NF- κ B homodimers. Bcl-3 may therefore aid activation of select NF- κ B-regulated genes, including those of the human immunodeficiency virus.

The structural similarity of the ankyrin repeat domains of Bcl-3 (ref. 1), I κ B (Mad-3) (ref. 3), pp40 (ref. 4), and the carboxy-terminal regions of the precursors for the p50 (I κ B- γ , pDI) and p50B subunits of NF- κ B^{2,5–12} prompted us to investigate the potential role of Bcl-3 in the regulation of NF- κ B activity. We have developed a transient transfection system with the human embryonic carcinoma cell line N-Tera-2, which has no detectable NF- κ B activity in uninduced cells¹¹. Expression of a CAT gene driven by the κ B sites of the human immunodeficiency virus (HIV- κ B) was entirely dependent on cotransfection with plasmid constructs encoding NF- κ B proteins (Fig. 1). The p65 subunit of the biochemically defined p50/p65 NF- κ B heterodimer^{13–15} could transactivate the reporter to generate very high CAT activity even when transfected alone, as noted previously^{10,16,17} (Fig. 1a); lower levels of p65, however, only weakly transactivated the reporter and required

synergy with cotransfected p50 for high activity (Fig. 1b). We observed dramatic concentration-dependent effects on transactivation with p65/p50 cotransfections. Although good synergy of p65 and p50 occurred with amounts of transfected p50 equivalent to or slightly higher than those of p65, more p50 dramatically decreased expression of the CAT reporter. These results agree with a previous report¹⁷ and they indicate that an excess of p50 led to the formation of p50 homodimers which inhibited the transactivation mediated by p65, presumably by competing with p50/p65 heterodimers for κ B sites. This interpretation is supported by mobility-shift experiments with nuclear extracts from cells that expressed increasing amounts of exogenously introduced p50 and a constant amount of exogenous p65 (Fig. 1c). The resulting increase in p50 homodimers progressively replaced the p50/p65 heterodimers from the κ B probe. As previously noted^{10,11,16–18}, p50 homodimers alone could not transactivate, regardless of the amount transfected, although a potentially different picture emerges from *in vitro* transcription experiments^{19,20}. Our data indicate that p50 homodimers could act as physiological inhibitors of NF- κ B-like activity, a conclusion also reached in a different experimental setting²¹.

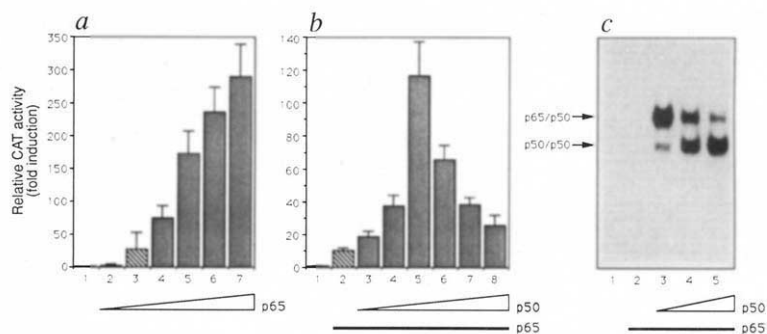
As Bcl-3 is a potential I κ B-like factor², we investigated whether Bcl-3 regulated κ B-dependent transactivation *in vivo*. Bcl-3 inhibited transactivation mediated by p50/p65 in N-Tera-2-based transient transfections, but only at very high concentrations compared with I κ B (Fig. 2a). Lower concentrations of Bcl-3 actually resulted in a small but consistent increase in transactivation. This increase became very dramatic when inhibiting conditions of high molar ratios of p50 compared with p65, were used (Fig. 2b, c): increasing levels of Bcl-3 completely reversed the inhibitory effects exerted by excess amounts of p50 in a dose-dependent manner. This effect of Bcl-3 was observed with several κ B reporters, including the HIV- κ B-driven CAT and the entire HIV long terminal repeat (LTR)-driven CAT; this natural promoter/enhancer responded even more strongly (Fig. 2b and c, respectively). With intermediate levels of Bcl-3 we observed high transactivation of the HIV- κ B-driven reporter, comparable to the highest activity seen when p50 and p65 synergized optimally in the absence of Bcl-3 (compare Figs 1b and 2b). At higher levels of Bcl-3, even the heterodimeric complexes were inhibited (Fig. 2a, b). In contrast, the HIV-LTR-driven activity continued to increase with the higher levels of Bcl-3, possibly reflecting additional activity of Bcl-3 in this natural promoter (Fig. 2c). We propose that the repressive p50 homodimers were a functional target of Bcl-3, Bcl-3 preferentially acted as an anti-repressor in our assays. I κ B, which also contains ankyrin repeats, did not act in this way (data not shown). Transfection of increasing amounts of Bcl-3 alone, or together with either p50 or p65 (concentrations as in Fig. 2) did

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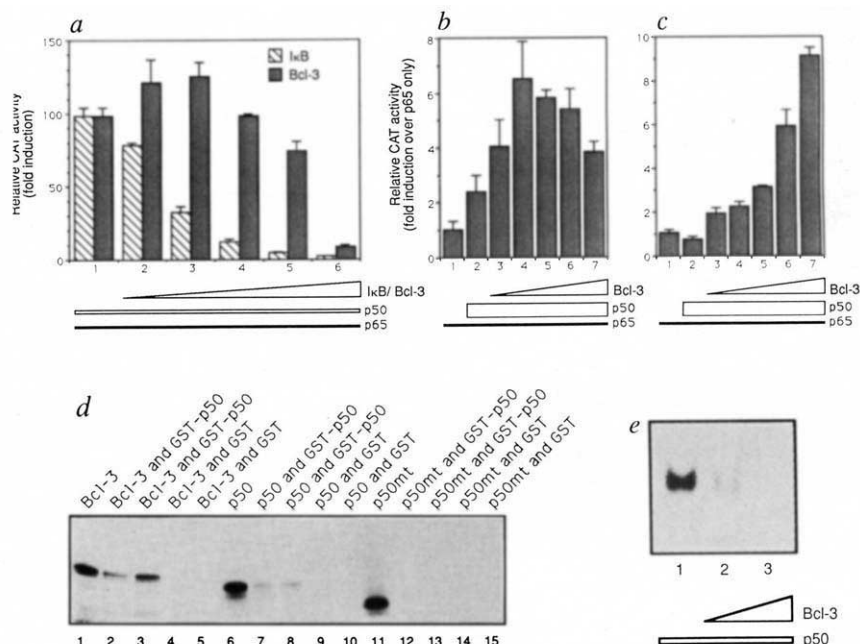
FIG. 1 Transactivation of an HIV- κ B-CAT reporter plasmid by transfection of NTERA-2 cells with expression vectors encoding *a*, p65 alone or *b*, p65 and p50. *a*, *b*, Show the fold induction of CAT activity over the activity produced by transfection of the reporter alone. The fold-induction values represent the mean of three or more independent transfections after normalizing to the protein concentrations of the cellular extracts. The total concentration of the transfected DNA and of the expression vector were kept constant throughout by adding appropriate amounts of expression vector without insert. Qualitatively similar results were obtained with an HIV-LTR-CAT reporter plasmid. *a*, Column 1, HIV- κ B-CAT reporter alone (6 μ g); 2–7, CAT reporter plus increasing amounts of the p65 vector, 0.01 μ g, 0.03 μ g, 0.1 μ g, 0.3 μ g, 1 μ g and 3 μ g, respectively. The fold induction over the HIV- κ B-CAT reporter cotransfected with 0.03 μ g of p65 vector (hatched) (see also ref. 16) is the reference point for the subsequent experiments in Fig. 2. *b*, Column 1, HIV- κ B-CAT (6 μ g); 2, HIV- κ B-CAT plus 0.03 μ g of p65 vector; 3–8, vectors as used in column 2 plus increasing amounts of the p50 vector, 0.01 μ g, 0.03 μ g, 0.1 μ g, 0.3 μ g, 1 μ g and 3 μ g, respectively. *c*, Increasing amounts of p50 homodimers displace p50/p65 heterodimers from a κ B site in electrophoretic mobility shift assays. Nuclear extracts were prepared from NTERA-2 cells transfected with: lane 1, the expression vector alone (without insert; 8.5 μ g); lane 2, the p65 vector (0.5 μ g); lanes 3, 4 and 5, the p65 vector (0.5 μ g) and the p50 vector (0.5, 4 and 8 μ g, respectively). As before, the total amount of the expression vector was kept constant. Our conditions preclude detection of binding by p65 homodimers. Identification of the complexes was accomplished by transfection of



p50 alone and by use of supershifting antibodies (see Fig. 4).

METHODS. The expression of p50 and p65 was directed by the expression vector PMT2T (ref. 31). The p50 construct contains the sequence of the p105 precursor from the ATG start codon to the *Xba*I site⁶⁻⁹. The reporter plasmids contain a CAT gene driven by the two κ B sites from the HIV virus inserted upstream of a minimal *c-fos* promoter (HIV- κ B-CAT)¹¹. The transfection procedure and the CAT assays were as described previously¹¹. NTERA-2 cells were lysed using a Dounce homogenizer and the resulting nuclei were salt-extracted according to standard protocols³². For electrophoretic mobility-shift assays 0.5 μ l of cell extract was incubated in a total of 10 μ l of buffer D (ref. 32) containing 1.3 μ g of poly dI-dC (Pharmacia), 1 μ g BSA, 0.02% Tween 20, and 0.035 ng of labelled probe (35,000 c.p.m.) for 30 min. The probe was the palindromic κ B probe described previously¹¹.

FIG. 2 Bcl-3 regulates NF- κ B activity. *a*, Bcl-3 inhibits transactivation mediated by cotransfected p50 and p65 in a dose-dependent manner, although it is much less effective than I κ B. Bcl-3 and I κ B (Mad-3) (ref. 3) expression was directed by PMT2T. Transfections were performed as outlined in Fig. 1. Columns 1–6, transfections with p50 (0.3 μ g), p65 (0.15 μ g), HIV- κ B-CAT reporter (6 μ g) and increasing amounts of cotransfected inhibitors I κ B (hatched bars) or Bcl-3 (dark bars); column 1, no inhibitors; 2, 0.03 μ g; 3, 0.1 μ g; 4, 0.3 μ g; 5, 1 μ g; 6, 3 μ g. *b*, Bcl-3 reverses inhibition mediated by high amounts of p50. The effect of increasing amounts of transfected Bcl-3 was measured under conditions similar to those described in Fig. 1b column 8 where high amounts of p50 inhibited transactivation by exogenously introduced p65 and p50/p65 complexes. The fold induction values are relative to the CAT activity obtained with 0.03 μ g of p65 vector (column 1). Column 1, HIV- κ B-CAT reporter (3 μ g) plus 0.03 μ g of p65; 2, HIV- κ B-CAT plus p65 (0.03 μ g) plus p50 (5 μ g); 3–7 vectors as used in column 2 plus increasing amounts of Bcl-3 vector, 1.5 μ g, 2.5 μ g, 3.5 μ g, 4.5 μ g and 5 μ g, respectively. *c*, An HIV-LTR-CAT construct³³ was used here instead of the HIV- κ B-CAT. Otherwise the experiments are identical to *b*. Bcl-3 had no effect on the expression of the transfected NF- κ B-encoding constructs as determined by western blotting of the p50 protein expressed from the transfected gene (data not shown). *d*, p50 associates with Bcl-3. ³⁵S-labelled Bcl-3 was produced by *in vitro* transcription/translation in reticulocyte lysates and subsequently incubated with bacterially produced glutathione S-transferase-p50 fusion proteins (GST-p50) attached to glutathione Sepharose 4B beads (for 1 h at room temperature in 150 mM NaCl, 20 mM Tris-HCl pH 7.4, 0.2% Triton X-100). The beads were precipitated and the attached material was separated on 10% SDS-polyacrylamide gels. The labelled wild-type and mutant p50 molecules were used as the positive and negative controls. The mutant p50 (p50mt) represents a deletion of the C-terminal end of the Rel-homology domain (deleted from the *Spe*I site)⁶ incapable of homodimerizing (unpublished observation) whereas the wild type will dimerize with a presumably limited number of monomers on the beads. Lanes 1, 6 and 11 represent 2 μ l of the *in vitro* translated material; 5 μ l were used for all other lanes. The bacterially



produced GST protein alone (5 μ g) was used as a negative control for the GST-p50 fusion protein (1.6 μ g). Lanes 2/3, 4/5, 7/8, 9/10, 12/13, 14/15 are duplicates of each other, except that the second of each pair contained beads that were pre-incubated with BSA before GST proteins were bound. *e*, Bcl-3 inhibits DNA-binding of p50 homodimers. Nuclear extracts (0.4 μ l) from NTERA-2 cells transfected with 5 μ g of the p50 expression vector were mixed with increasing amounts of whole-cell extracts of NTERA-2 cells transfected with 8 μ g of the Bcl-3 expression vector (0, 1.25 and 2.5 μ l, lanes 1, 2, and 3, respectively). The total amount of whole-cell extract was kept constant by adding appropriate compensating amounts of extract from NTERA-2 cells transfected with 8 μ g of the insert-less expression vector alone. The probe was the IL-2 Rec κ B probe described previously²⁶. Extracts were prepared as described in Fig. 1, except that cells were freeze-thawed for whole-cell extractions.

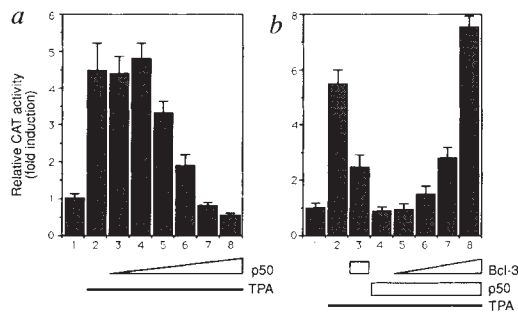


FIG. 3 p50 inhibits transactivation of the HIV-LTR-CAT reporter by endogenous NF- κ B, and Bcl-3 reverses this inhibition. N.Tera-2 cells were stimulated with TPA after transfection with the HIV-LTR-CAT reporter plasmid alone, or together with *a*, increasing amounts of p50 or *b*, a high amount of p50 and increasing amounts of Bcl-3. *a*, Column 1, HIV-LTR-CAT reporter plasmid (6 μ g), no stimulation; 2, HIV-LTR-CAT, TPA stimulation; 3–8, as 2, plus increasing amounts of p50, 0.05 μ g, 0.15 μ g, 0.5 μ g, 1.5 μ g, 5 μ g and 10 μ g, respectively. *b*, Column 1, HIV-LTR-CAT (6 μ g), no stimulation; 2, HIV-LTR-CAT, TPA stimulated; 3, HIV-LTR-CAT plus Bcl-3 (5 μ g), TPA stimulated; 4, HIV-LTR-CAT plus p50 (5 μ g), TPA-stimulated; 5–8, as 4, plus increasing amounts of Bcl-3, 0.15 μ g, 0.5 μ g, 1.5 μ g and 5 μ g, respectively. Transfected N.Tera-2 cells were stimulated with 10 ng ml⁻¹ of TPA for 6 h before collection.

not increase transactivation of the HIV- κ B-CAT reporter. Furthermore, the results were entirely dependent on the κ B sites, as a mutant κ B construct was never transactivated (data not shown).

Bcl-3 has been reported to decrease DNA-binding of p50 (ref. 2). We provide evidence for a physical association between Bcl-3 and p50. A glutathione *S*-transferase (GST) fusion protein of p50 was used to coprecipitate radiolabelled Bcl-3 on glutathione-coated beads, whereas a negative control was not precipitated (Fig. 2*d*). Furthermore, antibodies against Bcl-3 were able to coprecipitate p50 but not p65 out of cell extracts (V.B., G.F. & V.S. manuscript in preparation). Finally we demonstrated that

binding of p50 homodimers to DNA was progressively inhibited by increasing amounts of extracts from Bcl-3 transfected cells but not from untransfected cells (Fig. 2*e*). The individual ankyrin repeat domains present in the various I κ B-related proteins (described previously) may be responsible for their physical association with distinct but related proteins of the NF- κ B family; they may inhibit complex formation by shielding functionally relevant domains^{2,5,22–27}.

The inhibition by p50 and the subsequent reversal of this inhibition by Bcl-3 was observed also in activated cells. N.Tera-2 cells contain limited but detectable NF- κ B-like activity when stimulated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), possibly because of new synthesis of NF- κ B. Exogenously introduced p50 effectively inhibited the TPA-induced endogenous activity seen with the HIV-LTR in a dose-dependent manner (Fig. 3*a*). We observed a small amount of synergy at very low levels of p50 and an increasing suppression at higher levels of p50, as might be expected from the results discussed above. Increasing amounts of cotransfected Bcl-3 reversed the inhibition seen with high p50 levels in these activated cells (Fig. 3*b*). Qualitatively similar results were obtained with the HIV- κ B constructs (data not shown). Transfection of Bcl-3 alone (Fig. 3*b*) did not increase the TPA-induced transactivation; rather, it was suppressed (see Fig. 2*a*), confirming that the transfected p50 homodimers must be a functional target for Bcl-3 to cause transactivation.

The potential inhibitory role of p50 homodimers led us to investigate the natural occurrence of such complexes in primary cells. Previous data indicated that p50 homodimers may exist^{28,29}, but no direct evidence has been documented. We demonstrated an abundance of p50 homodimeric complexes in resting cells. Two distinct κ B-specific complexes were present in resting peripheral blood T cells (Fig. 4*a*, lane 1; competition with wild-type and mutant κ B sites, lanes 2 and 3, respectively). Use of supershifted antibodies against p50 (lane 4) and p65 (lane 5) allowed us to identify the faster migrating complex as containing predominantly p50 homodimers and the slower one

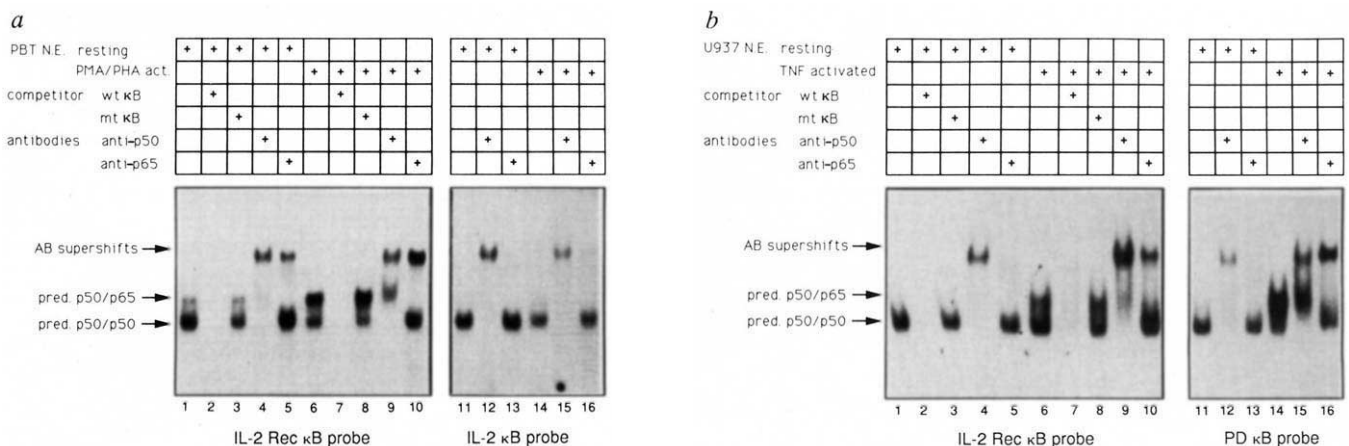


FIG. 4 Electrophoretic mobility-shift assays of nuclear extracts (NE) from resting or phytohemagglutinin (PHA)/TPA activated (act.) peripheral blood T (PBT) cells (*a*) and from resting or tumour-necrosis factor (TNF)- α activated U937 cells (*b*). Supershifts were performed with antibodies directed against the N-terminal 13 amino acids of p50 (ref. 6) and the N-terminal 14 amino acids following the initiator methionine of p65 (ref. 14). All complexes are marked by arrows (pred., predominant; AB, antibodies). The κ B probes used are indicated below the panels. Competitions were performed with a 90-fold excess of cold probes (wt, wild-type; mt, mutant; see later). The indicated assignment of the two complexes was supported further by the fact that they comigrated almost with known p50 homodimeric and p50/p65 heterodimeric complexes obtained using extracts from appropriately transfected N.Tera-2 cells (p50 truncation was close to the presumed natural processing region)²⁹. Use of these transfected cells revealed that the p50 antibody was less efficient in supershifting the p50/p65 heterodimeric complex than the homodimeric complex, indicating that the anti-p50 anti-

body-mediated supershift here underestimated the p50 content of the upper complex. We note the presence of some heterodimeric complexes in the resting peripheral blood T cells but not in several cell lines. On the basis of fluorescence-activated cell sorting analysis, we think it likely that blood obtained from different volunteers contained sufficient activated cells for detection with this assay (data not shown).

METHODS. Peripheral blood T cells were purified as described previously³⁴. T cells were stimulated with PHA (1 μ g ml⁻¹) and TPA (10 ng ml⁻¹) for 2.5 h and U937 cells were stimulated with TNF- α (Genzyme, 100 U ml⁻¹) for 2.5 h. To obtain nuclear extracts, T cells were lysed with NP-40 (0.2%) and U937 cells were lysed in a Dounce homogenizer. Preparation of nuclear extracts and mobility shifts were as Fig. 1, except that 0.2 ng of probe were used. For antibody supershifts 0.9 μ l of antisera were included as well. The probes have been described previously (PD κ B and mutant PD κ B¹¹, IL-2 Rec κ B²⁸ and IL-2 κ B and mutant IL-2 κ B⁶).

as containing predominantly p50/p65 heterodimers. Specific antibodies against p50B, RelB and c-Rel confirmed that these other proteins were either undetectable or only weakly present in the two complexes (data not shown; see legend to Fig. 4 for additional information). The relative amount of p50 and p50/p65 complexes visualized on gels depended partly on the actual κ B sequence used; the interleukin-2 (IL-2) κ B site, for example, strongly favoured p50 binding (lanes 11–16), which may have physiological consequences for IL-2 expression²¹. The immunoglobulin κ B site (core sequence identical to each HIV κ B site) gave results comparable to the IL-2 receptor site. Once cells were stimulated (Fig. 4a, lanes 6–10) the ratio of p50/p65 over p50/p50 dramatically increased (compare lanes 1 and 6). We also noted a decrease of the p50 homodimeric complex on activation of T cells which may be due to Bcl-3. A similar activation-dependent decrease in p50 homodimers has recently been reported for mouse T cells²¹. Figure 4b demonstrates the presence of p50 homodimeric complexes in U937 cells, in an analysis similar to the one shown for T cells. These data are consistent with, but do not prove, a role of p50 homodimers in inhibiting κ B-dependent transcription in resting cells. Activation of the resting cells thus shifts the balance in favour of the transactivating NF- κ B complexes, the degree of the shift depending on the actual κ B sequence.

Our data reveal an unexpected function for Bcl-3. This putative oncoprotein may aid the activation of certain NF- κ B-regulated genes and may play a role in the pathophysiology of HIV. The translocated and consequently inappropriately expressed Bcl-3 gene may contribute to chronic lymphocytic leukaemias by deregulating NF- κ B activity, as may the c-Rel and p50B genes when chromosomally rearranged^{12,30}. Bcl-3 may shift the balance between inhibition and activation of certain κ B-site-regulated target genes.

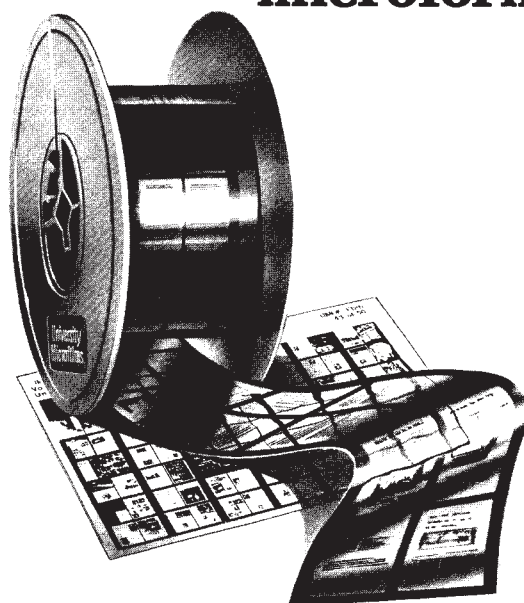
Note added in proof: A recent report³⁵ also demonstrates in detail the physical interaction of Bcl-3 with p50/NF- κ B. □

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- Ohno, H., Takimoto, G. & McKeithan, T. W. *Cell* **60**, 991–997 (1990).
- Hatada, E. N. *et al. Proc. natn. Acad. Sci. U.S.A.* **89**, 2489–2493 (1992).
- Haskill, S. *et al. Cell* **65**, 1281–1289 (1991).
- Davis, N. *et al. Science* **253**, 1268–1271 (1991).
- Inoue, J.-I., Kerr, L. D., Kakizuka, A. & Verma, I. M. *Cell* **68**, 1109–1120 (1992).
- Bours, V., Villalobos, J., Burd, P. R., Kelly, K. & Siebenlist, U. *Nature* **348**, 76–80 (1990).
- Ghosh, S. *et al. Cell* **62**, 1019–1029 (1990).
- Kieran, M. *et al. Cell* **62**, 1007–1018 (1990).
- Meyer, R. *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 966–970 (1991).
- Schmid, R. M., Perkins, N. D., Duckett, C. S., Andrews, P. C. & Nabel, G. J. *Nature* **352**, 733–736 (1991).
- Bours, V. *et al. Molec. cell. Biol.* **12**, 685–695 (1992).
- Neri, A. *et al. Cell* **67**, 1075–1087 (1991).
- Nolan, G. P., Ghosh, S., Liou, H.-C., Tempst, P. & Baltimore, D. *Cell* **64**, 961–969 (1991).
- Ruben, S. M. *et al. Science* **251**, 1490–1493 (1991).
- Urban, M. B., Schreck, R. & Baeuerle, P. A. *EMBO J.* **10**, 1817–1825 (1991).
- Ruben, S. M., Narayanan, R., Klement, J. F., Chen, C.-H. & Rosen, C. A. *Molec. cell. Biol.* **12**, 444–454 (1992).
- Schmitz, M. L. & Baeuerle, P. A. *EMBO J.* **10**, 3805–3817 (1991).
- Ryseck, R.-P. *et al. Molec. cell. Biol.* **12**, 674–684 (1992).
- Fujita, T., Nolan, G. P., Ghosh, S. & Baltimore, D. *Genes Dev.* **6**, 775–787 (1992).
- Kretzschmar, M., Meisterernst, M., Scheidereit, C., Li, G. & Roeder, R. G. *Genes Dev.* **6**, 761–774 (1992).
- Kang, S.-M., Tran, A.-C., Grilli, M. & Lenardo, M. J. *Science* **256**, 1452–1456 (1992).
- Baeuerle, P. A. & Baltimore, D. *Science* **242**, 540–546 (1988).
- Kerr, L. D. *et al. Genes Dev.* **5**, 1464–1476 (1991).
- Blank, V., Kourilsky, P. & Israël, A. *EMBO J.* **10**, 4159–4167 (1991).
- Henkel, T. *et al. Cell* **68**, 1121–1133 (1992).
- Lux, S. E., John, K. M. & Bennett, V. *Nature* **344**, 36–42 (1990).
- LaMarco, K., Thompson, C. C., Byers, B. P., Walton, E. M. & McKnight, S. L. *Science* **253**, 789–792 (1991).
- Molitor, J., Walker, W., Doerre, S., Ballard, D. W. & Greene, W. C. *Proc. natn. Acad. Sci. U.S.A.* **87**, 10028–10032 (1990).
- Rivière, Y., Blank, V., Kourilsky, P. & Israël, A. *Nature* **350**, 625–626 (1991).
- Lu, D. *et al. Oncogene* **6**, 1235–1241 (1991).
- Israel, D. I. & Kaufman, R. J. *Nucleic Acids Res.* **17**, 4589–4604 (1989).
- Dignam, J. D., Lebovitz, R. M. & Roeder, R. C. *Nucleic Acids Res.* **11**, 1475–1489 (1983).
- Gendelman, H. E. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 9759–9763 (1986).
- Zipfel, P. F., Irving, S. G., Kelly, K. & Siebenlist, U. *Molec. cell. Biol.* **9**, 1041–1048 (1989).
- Wulczyn, F. G., Naumann, M. & Scheidereit, C. *Nature* **358**, 597–599 (1992).

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The computing edge

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IBM has extended the performance and versatility of its **POWER visualization system** by boosting its processing power, memory and functionality (*Reader Service No. 100*). In addition, the company has enhanced its Visualization Data Explorer software and the availability of versions that run on other manufacturers' workstations. The improvements are intended to speed and ease the creation of visual images — images derived from large calculations that can be manipulated, made to move and explored. Major changes include: improved software, open systems advantages, a more powerful server, more memory and a multi-user capability. The improvements to Data Explorer include an enhanced data import facility, an improved user interface and enhanced module functionality. Prices for the IBM POWER visualization server start at \$320,000 (US). The price for all versions of the IBM Visualization Data Explorer — whether available for IBM RISC System/6000 or other manufacturers' workstations — is \$5,900 (US), including maintenance.

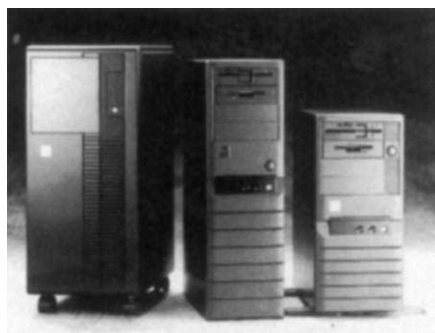
■ Hardware options

Weighing only 1 kg and the size of a paperback book, the new MicroLight IIIsx **personal computer** from MicroLight Systems is designed to be carried in a pocket or a briefcase and provides new levels of flexibility to users of personnel computers (*Reader Service No. 101*). The MicroLight IIIsx includes 4 Mb of RAM, a 60-Mb hard disk, a 3.5-inch floppy disk and colour super VGA graphics as standard; a maths co-processor is optional. The 386sx main processor runs at 25 MHz, making the IBM-compatible MicroLight IIIsx capable of running even the most demanding software such as Windows 3.0 and AutoCAD. The MicroLight IIIsx is designed to be used in conjunction with either colour or black and white monitors and standard keyboards. When leaving work in the evening, the user unplugs the base unit, slips it into a pocket or briefcase and then plugs it into a spare screen and keyboard at home. Alternatively, users can lock the base unit away at night in a drawer or fire-proof safe/filing cabinet to provide added security for important data. The complete £1,795 (UK) system comes with a 14-inch super VGA monitor and a 102-key keyboard; the base unit alone costs £1,545 (UK).

Sun Microsystems has unveiled its next generation of **desktop workstations** — the SPARCstation 10 line — aimed at professionals in both the commercial and technical markets who need fast application per-

formance (*Reader Service No. 102*). With this new computer, the company has redesigned the SPARCstation architecture, taking a new approach to the memory, bus, disk and networking subsystems that significantly boosts application performance. Built around the fast new superscalar SuperSPARC chip from Texas Instruments, the SPARCstation 10 achieves a multiprocessing performance rating of up to 218 (SPECthruput89) and more than 400 MIPS in its four-microprocessor configuration. Its modular design is intended to provide a cost-effective upgrade path to higher performance — and multiple — microprocessors. The company says that the design of the SPARCstation 10 has been tuned to maximize application performance even when the system is running under the most demanding conditions. The new SuperSPARC CPU delivers two-to-four times the performance of previous SPARC microprocessors. Sun Microsystems has quadrupled the speed at which data can be processed through the memory on the SPARCstation 10 and has more than doubled the rate at which the system can retrieve data from disks. Bus speed — the rate at which the system can send and receive information from peripherals — has been doubled over previous SPARCstation systems. The SPARCstation 10 is also a RISC workstation with a 1-Mb external memory cache, called SuperCache, which is designed to provide an added boost to application performance. Four models of the SPARCstation 10 are available with prices ranging from \$18,495 (US) to \$57,995 (US).

Microway has launched its new **486 workstation series** — a range of customized systems designed to cater for computationally-intensive scientific applications such as chemical modelling, quantum chemistry, data collection and software development (*Reader Service No. 103*). The workstations, which can be readily expanded



Microway's scientific workstation range.

and enhanced in line with user demand, are able to house file servers, i860 arrays and any combination of peripherals or co-processors that place large demands on power, space and cooling. The systems are based on a 486-powered EISA motherboard, which has the capacity to tie together high-speed communications, data retrieval and data processing. Users can dictate the motherboard specifications, tower size and memory configuration and can choose to have an optional Weitek numerical co-processor or Number Smasher-860 installed. Microway says that a distinguishing feature of the workstation range is its across-the-board cooling system that draws cool air across hot spots on the bus, thus eliminating the principal cause of computer failure. Prices start at £1,295 (UK).

Leabrook Computing announces a range of **personal supercomputers** based on the personal computer and the Intel i860 vector microprocessor (*Reader Service No. 104*). The company has combined the best of these technologies to provide complete turnkey systems for engineers, chemists,



Personal supercomputers from Leabrook.

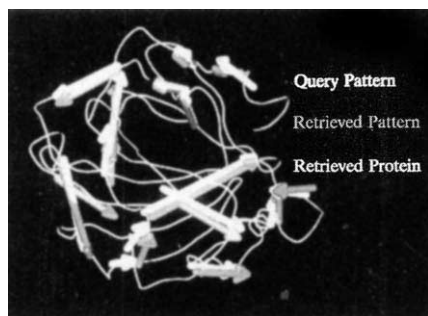
physicists for massive databases and for image processing. Leabrook says that the maximum benefit from the inexpensive i860 is obtained through its parallel implementations from Transtech Parallel Systems. Being parallel, the company says it can offer peak speeds from 60 million floating point operations a second (Mflops) to one Giga-flop (1,000 Mflops) in desktop and desktop systems. The i860-based Superstation graphics board from Hercules now provides photo-real colour and three-dimensional manipulation speed needed for visualization of the results from these systems. A full range of numerical, mathematical, statistical, graphical and image processing software is available to support the porting or development of applications. The line-up of products includes the PAX portable supercomputer, the QIX desktop super-

PRODUCT REVIEW

computer, PARAstation systems and Gigaflop systems. Prices range from £9,750 (UK) to £89,950 (UK).

■ Protein research

Tripes is now distributing PROTEP, the software for **protein motif searching and discovery** developed by Peter Willett, Pete Artymiuk and David Rice at the University of Sheffield (*Reader Service No. 105*). With PROTEP, and its companion package, COMPOSER, researchers are provided with



Tripes' new PROTEP protein motif searching and discovery software.

a complete protein modelling tool set, which is available under a unified interface system. It allows protein crystallographers to identify quickly all known protein structures that have three-dimensional (3D) features in common with a newly solved structure. The program's two-part algorithm technique identifies common secondary structure motifs and degrees of relatedness in proteins by searching a 3D database of known protein structures. PROTEP's Ullman subgraph isomorphism technique locates all proteins in the Brookhaven Protein Data Bank that contain a given 3D structural motif defined in terms of alpha-helices and beta-strands. The program is designed to accept partially defined structures, which, Tripes says, can be of great value when searching for hypothetical motifs, or when full 3D coordinates of the proteins are not available. PROTEP's method of 'clique' detection for protein

motif discovery submits a complete protein structure to find all common subpatterns (cliques) between the designated protein and all others in the Protein Data Bank. The program thereby highlights interesting structural similarities that may not have been previously noted or suspected. When used in combination with COMPOSER (Tripes's homology modelling module), the user can generate a fully atomistic structural model to compare with experimental data. PROTEP is available on Silicon Graphics' IRIS platforms either for use with SYBYL or as a standalone package.

Blue Lightning Data and Software has introduced a new **protein database**, BioAlmanac, which can be used to identify unknown proteins and to plan protein purification protocols (*Reader Service No. 106*). BioAlmanac contains purification results data as well as biochemical experimental data such as molecular weights (as measured by any method), isoelectric points, subunit compositions, carbohydrate contents, biological functions and amino acid compositions. It is integrated with the PIR, SWISS-PROT and GenBank databases to provide the user with multiple database search capabilities. Searches can be performed using specific data field indexes or a global full-text index. Reference tables with protein and organism synonyms, EC numbers and product specifications are included to ensure comprehensive searching, the company says. An IBM PC-compatible CD-ROM contains both the databases and search software — no hard disk is required. BioAlmanac version 1.0 focuses on human blood proteins, receptors, DNA binding proteins and regulatory proteins.

Two Silicon Valley-based companies have introduced a **molecular biology software system** that is designed to reduce dramatically excessively long sequence matching calculations — from about 10 hours to less than 30 seconds (*Reader Service No. 107*). IntelliGenetics and MasPar have introduced BLAZE software for the large-scale comparison of biological

sequence data using MasPar's MP-1 family of massively parallel computers. The software supports biological sequence similarity searching using an optimized implementation of the Smith-Waterman sequence comparison algorithm. An electronic mail interface using the standard SMTP protocol allows operation of the system either as a local-area or a wide-area network server to support a large number of users. The MP-1 system running BLAZE performs sequence database searches and comparisons about one thousand times faster than a workstation, say the companies. For example, BLAZE on the MP-1 needs only 28 seconds to compare an average protein to an entire database of 16,941 protein sequences using the Smith-Waterman method. MasPar says that this single query when carried out with the same stringency of search on a typical workstation would have taken about 10 hours. The \$20,000 (US) BLAZE software will support searching of both DNA and protein sequence databases and will identify both local and global homologies (strongly similar portions of the sequences) by allowing the user to change the gap penalty used by the scoring algorithm.

■ Dealing with data

PeakFit 3.0 from Jandel Scientific is a major update to the company's **chromatography/spectroscopy analysis software** package for IBM PC compatibles (*Reader Service No. 108*). Jandel Scientific says that, unlike other analysis techniques, PeakFit



PeakFit 3.0 — nonlinear curve-fitting software for the PC.

uses advanced nonlinear curve fitting to reduce effectively noise and separate and characterize unresolved peaks in overlapping peak data. Analysis includes all model parameters and statistics, in addition to system suitability information critical to chromatography (peak area, asymmetry, resolution, column efficiency/number of theoretical plates) and characterization data useful in spectroscopy (peak amplitude, area, centre, width). Besides utilizing nonlinear fitting for more precise peak deconvolution, the \$595 (US) PeakFit program also offers the ability to observe and control the nonlinear fitting process graphically onscreen. Version 3 adds several new features including more built-in functions,

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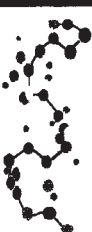
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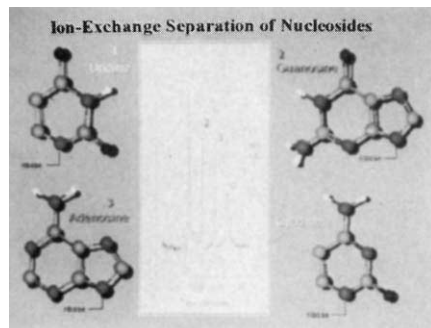
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Abacus Concepts is shipping copies of StatView 4.0, the company's integrated **data analysis and presentation graphing system** for the Macintosh (*Reader Service No. 109*). StatView 4.0 allows researchers to manage and analyse their data, and then graph and create presentations of their results all within a single application. This eliminates the need for four separate applications (spreadsheet, statistics, graphing and drawing). At the heart of StatView's integrated system, Abacus says, is powerful data analysis. The integrated analysis environment provides two new tools for building analyses: the analysis browser, which lists all available analyses, including both statistics and graphs; and the variable browser, which is a floating palette that connects the user to data. With it, the user can choose which variables to analyse from any dataset or combination of datasets. Once the analyses and variables have been selected, all the results appear in the 'view' window — a complete drawing and analysis environment. Each view can contain as many pages as the user wishes (up to 638) and as many results as the user would like. In the view window, the user can combine tables and graphs with drawn objects and text, creating an entire presentation. StatView 4.0 costs \$595 (US); owners of StatView 2.0 can upgrade for \$149 (US).

TriMetrix has introduced version 2.0 of Axum, the company's **scientific graphics and data analysis package** for PCs (*Reader Service No. 110*). This latest version allows users to automate repetitive graphing and data analysis tasks with extensive batch processing capabilities. For example, researchers can now have Axum automatically load new data and update a set of graphs on a regular basis. These batch programs can be written automatically by Axum and can even be used to control Axum from other programs. In addition, Axum 2.0 offers all-new automatic axes scaling and tick placement methods that allow users to create publication-quality graphs more efficiently. TriMetrix says that Axum also offers major advances in three-dimensional (3D), two-dimensional (2D) and contour plotting. Axum users can now use unlimited-sized data sets to produce 3D mesh surfaces, which can be filled with varying colours. In Axum 2.0, stacked 3D contours can be created for both gridded or irregularly spaced data. Enhanced 2D graphing capabilities include additional curve-fitting plot types and labelled scatter plots. Users can also choose from PostScript fonts in addition to the 22 built-in fonts when annotating their graph. New supported graphics

export file types include TIFF, Colour PostScript and HPGL2. Axum's data editor lets users sort multiple columns of data of any size. Axum 2.0 costs \$495 (US); registered Axum users can upgrade for \$99 (US).

Last month, Molecular Arts Corporation announced the release of version 1.1 of chemEXHIBIT, a desktop tool for the **illustration and presentation of chemical research** (*Reader Service No. 111*). The



chemEXHIBIT — a desktop tool for chemical research illustration/presentation.

program offers documentation, illustration and presentation capabilities that allow chemists to display, manipulate and render three-dimensional (3D) molecular structures, create two-dimensional (2D) chemical diagrams, create publication-quality graphic illustrations, import graphic images from third-party applications, document the results of their research and produce materials for scientific presentations. Included in the \$595 (US) chemEXHIBIT package is a full suite of 2D diagram drawing tools, CHEMGRAM, for creating 2D pictographs of chemical structures. CHEMGRAM comes with 39 diagram drawing tools for drawing bonds, rings, orbitals and atom labels; snap-to drawing options; adjustable position, angle and bond length constraints; and automatic superscripts and subscripts in labels. Users can also read in 3D structure models from third-party databases or modelling programs. The VISAGE-3D rendering tools enable chemists to create the look they need to illustrate important scientific issues. The VISAGE-3D toolset includes mixable rendering styles (wire frame, ball and stick, space fill and cylinder) with support for depth cueing, perspective, shading and stereo images. When used with the SELECT-3D substructure selection tools, chemists can rapidly modify their visuals to suit their documentation needs. Additionally, the TAGIT-3D tools allow the user to identify and tag important structural and chemical attributes of 3D models. □

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